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THE DIAGNOSIS OF UNSUSPECTED CANCER OF THE CERVIX[1]

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In recent years interest has been growing in the detection of early malignant disease. This interest coupled with new diagnostic techniques has brought about the development of the "Cancer Detection Clinic." One of these clinics was established in November 1947 by the Cancer Control Division of the National Cancer Institute under the direction of Dr. Austin V. Deibert. This clinic was set up in conjunction with a previously established treatment center for venereal disease in Hot Springs, Arkansas.

All females in the cancer age group admitted to the venereal disease center were routinely given an examination directed primarily toward the diagnosis of malignant disease. This routine offered a somewhat unique opportunity to examine patients in which suspicion of cancer played no part in determining their admission to the center. The group was characterized, however, as having a high incidence of syphilis (91 percent), largely Negro (93.2 percent), and of low economic status, which might have some influence on the incidence of cancer found and tissue-site distribution.

Out of the first 3,224 female patients routinely examined, 67 primary cervical carcinomas were diagnosed by histologic study, most of these at a very early stage. This paper is concerned with these cases and their diagnosis.

In this study the biopsy specimens on 32 cases fulfilled the microscopic criteria for a diagnosis of carcinoma *in situ*— or intraepithelial carcinoma of the cervix. Before further discussion it is best to clarify the interpretation of this entity.

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As used by the pathologist who made the histologic diagnoses [4], carcinoma *in situ* refers to a limitation of the neoplastic cells by the basement membrane of the surface epithelium or of the cervical glands. Even though the glands may be filled with neoplastic cells, unless there is a demonstrable break through the basement membrane the classification is carcinoma *in situ*. This definition is in accord with that of Foote and Stewart (*1*), Pund and Auerbach (*2*), and Gurskis, Beaver, and Nelson (*3*).

The definition of histologic carcinoma *in situ* is comparatively easy, but what this finding means in terms of actual malignant potential is not definitely established. The precise point at which these malignant-appearing localized cells achieve the irreversible characteristics of cancer apparently cannot be determined by cell morphology alone.

There is evidence that some of the carcinomas *in situ* will eventually invade and become malignancies of the higher grade, but the reported number of cases which have been observed to do this is small (*4*, *5*). The relatively small number of malignancies diagnosed in this early phase have, for the most part, been subjected to treatment of some sort and therefore the evaluation of their outcome is made difficult. Similarly, there is little accord as to what constitutes adequate treatment for these early lesions. Owing to the serious nature of cancer in general, it is deemed wiser to treat the lesion with the most radical procedure compatible with the patient's age and ovarian function.

In our series of 32 intraepithelial carcinomas we do not have the diagnosis confirmed by serial sections of the cervix, and lacking this, the possibility of focal invasion of the stroma in some cases cannot be denied. However, to support the plausibility of a diagnosis of carcinoma *in situ* is the fact that the average number of sites biopsied is 3.5 per case without evidence of invasion.

Illustrations of the histologic pattern of the cases of carcinoma are seen in the photomicrographs of four cases. In each case, the diagnosis was carcinoma *in situ* (figs. 1 and 2).

Since cancer still limited to the cervix varies greatly in its clinical and histologic picture as well as in its prognostic implications, these cases (Stage I, League of Nations standard clinical grading) are divided into 3 subgroups. The group of 32 cases diagnosed histologically as being intraepithelial carcinoma of the cervix is designated as Stage IA. Another group of cases, of which there are 7, with microscopic evidence of minimal focal penetration of cancer cells into the stroma is called Stage IB, and another group of 7 cases with frankly invasive carcinoma still limited to the cervix is called Stage IC. The remaining 21 cases of cervical carcinoma were clinically advanced beyond the confines of the cervix and are classified as either Stage II, Stage III, or Stage IV, and considered as a unit in tables 1 and 2.

Our program called for routine examination for cancer of all female patients in the cancer age group admitted to the Hot Springs Medical

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[4] The histologic study of this material was made by Chapman H. Binford, M. D., Pathologist, U. S. Marine Hospital, Baltimore, Md., whose assistance is gratefully acknowledged.

Center for diagnosis and treatment of venereal disease. These patients were brought to the center in buses from scattered areas throughout the State of Arkansas and the special cancer examination was arranged in a brief schedule so as not to prolong the routine stay for venereal disease care. Because of this situation and the variation in patient load it was found necessary at times to adjust arbitrarily the minimum age of those examined from 35 to 40 years. This necessity unfortunately precluded the diagnosis of possible cases occurring at a younger age.

In the 63 cases of intraepithelial carcinoma diagnosed by examination of surgical specimens by Pund *et al.* (*6*) the average age was 37.9 years, and in 66 more cases diagnosed by biopsy by the same group the average age was 40.8 years. In 11 cases reported by TeLinde and Galvin (*7*) the average age was 36 years. These authors considered the involvement of cervical glands in 10 of these cases as invasion, which differs from our interpretation as described above. Foote and Stewart (*1*) presented 24 cases with an average age of 39.0 years.

The establishment of a minimum age limit for the group we examined places limitations on the comparison of our cases with these figures. During the period when the minimum age was set at 35 years the intraepithelial carcinomas diagnosed had an average age of 40.6 years (table 1). The Stage IB and IC cases gave a combined average age of 43.7

TABLE 1.—*Mean ages for cervical cancer cases by stage of disease*

Stage of disease	All cancer cases	35-year lower-age limit
IA	42. 5	40. 6
IB and IC	43. 2	43. 7
II, III, and IV	48. 2	47. 4
All stages	45. 6	----------

years, and the Stage II, III, and IV patients averaged 47.4 years. Including all cases (where the minimum age was set at 35 and 40), the Stage IA patients averaged 42.5 years, Stage IB and IC 43.2 years, and the clinically advanced cases 48.2 years. The average age of all 3,224 cases examined was 45.6 years. While these percentages are not significantly different because of the small numbers of cases and wide scatter of ages about the mean age in each class, they conform to the findings of other investigators.

Despite the exclusion of possible cases below age 35, when this was the minimum age for those examined, there is an interval of 3 years between the mean age of patients with a preinvasive (intraepithelial) stage and the early infiltrative cases, and approximately 7 years between the preinvasive and clinically advanced cases. This points up the importance of the presymptomatic, incipient stage of cervical cancer and the possibility of diagnosing it over a period of several years before it becomes clinically apparent. Taylor and Guyer (*8*) have reported a case showing early carcinomatous infiltration of the cervix in 1938 that was still limited

to the cervix in 1945. Schiller (*9*) also reported finding an early infiltrative cervical lesion on a surgical specimen and the patient had evidence of small metastases 4 years later.

The patients with cervical malignancies had an average of 2.9 pregnancies with term deliveries compared with 2.6 for the entire group of patients examined. Although the cancer group had a slightly higher average number of pregnancies, there were 11 nulliparous patients in this group as well as 18 primiparas. Nine patients with cancer had more than 5 deliveries, the largest number per individual being 16.

Abortions were frequent in this group of patients. The high incidence of syphilis plus the frequent finding of pelvic disease of all types helped to account for this. To total the number of abortions accurately proved impossible because of the tendency on the part of the patients to consider any menstrual irregularity as a "miscarriage."

The menstrual histories in the cancer group were noteworthy only in the lack of suggestive symptoms in the early stages. In general, the group of patients examined gave a history of an early menarche and menopause. Cessation of menses in the fourth decade was not unusual.

Only one of the intraepithelial carcinoma cases presented a history of menorrhagia and metrorrhagia although two others had noted a diminution in amount and frequency of menses attributed to the menopause. Two of the cases with cervical cancer in the early, focal, infiltrative stage had noted a slight increase in menstrual flow, one for the preceding year and one for 5 months. Two Stage IC cases had menstrual irregularities. One had skipped periods occasionally for 3 years and another had menorrhagia of a moderate degree for 3 months.

Complaints referable to the female pelvic organs were frequent in all patients, and those with early cervical malignancies rarely presented symptoms of practical aid in their diagnosis. Of the entire group of 3,224 patients included in this survey, 21.9 percent stated they had leukorrhea of some degree. Frequently, physical examination revealed the presence of a profuse mucoid discharge that was not admitted in the history. Fifteen of the intraepithelial carcinoma cases gave a history of vaginal discharge of some sort and although this is double the general incidence, it in no way aided in the diagnosis of a specific malignancy. In the Stage IB group, 3 cases complained of leukorrhea, for an incidence of 42.8 percent. All 7 of the Stage IC cancer cases, and 19 of the 21 cases with more advanced stages of the disease, stated that they had a vaginal discharge.

Vaginal spotting was also rare in the early malignant cases and was present in only one Stage IA case, and that was minimal in amount and occurred only once. One patient in the Stage IB category stated that she had noted occasional light bloody spotting for 3 weeks prior to examination. Oddly enough, all the Stage IC cases denied vaginal bleeding. Only one of the cases in the advanced cancer group had a negative menstrual and gynecologic history.

Thus it can be seen that the frank symptoms of cervical cancer are of aid in diagnosis in most cases only when the optimum time for treatment of the disease is gone.

Although the clinical examiners were not trained gynecologists, it is significant that despite the fact that they were searching specifically for early carcinoma and had the opportunity to see a relatively large number of very early malignant lesions, none professed to have the ability to discriminate between benign and early malignant conditions of the cervix to any practical degree. This is borne out by the fact that only four of the intraepithelial lesions were suspected clinically, and in no case did the examiner make an unequivocal diagnosis of malignancy (table 2).

TABLE 2.—*Initial clinical, biopsy, and cytologic evaluation of cases by stage of disease*

Stage	Total number cases	Clinical suspicion of cancer (initial examination)		Initial biopsy reveals cancer or suspicion		Initial cytologic test indicates cancer or suspicion		Cases biopsied only after positive cytologic test		Accuracy of biopsy when performed on clinical indication	
		Number	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
Stage IA (*in situ*)	32	4	12. 5	28	87. 5	28	87. 5	5	15. 6	23	71. 9
Stage IB and IC	14	8	57. 1	13	92. 9	9	64. 3	2	14. 3	11	78. 6
Frank clinical cancer stage II, III, and IV	21	21	100	[1] 19	100	13	[2] 68. 4	0	0	[1] 19	100
Total	67	33	49. 3	[3] 60	92. 3	50	[3] 76. 9	7	10. 4	[3] 53	81. 5

[1] Biopsy not done on 2 cases.
[2] No interpretation on 2 cases due to bloody smear.
[3] Total of 65 cases.

This represents only 12.5 percent of these cases. In the group of seven Stage IB cases, carcinoma was suspected on initial examination in three cases, or 42.8 percent; while in the seven Stage IC cases, carcinoma was suspected in five cases, or 71.4 percent.

In the remaining 21 cases of Stage II, III, and IV carcinomas, the clinical diagnosis was correct in each case. This latter group represents the preponderance of cases that present themselves with symptoms of cervical cancer to the doctor (table 2).

Pathologic findings other than cancer on pelvic examination were frequent. There were 423 cases of uterine fibroids diagnosed by physical examination out of the 3,224 patients. This is an incidence of 13.1 percent. A total of 7 of the 67 cancer cases had fibroids, for an incidence of 10.4 percent.

There were 124 benign cervical polyps noted in the total group examined, for an incidence of 3.9 percent. Six of these were in the patients with cervical cancer, for an incidence of 9.0 percent. In no case was there microscopic evidence of malignancy in the polyp itself. This points up a frequent error in which the clinician removes a polyp for histologic study and fails to include tissue from the base of the polyp which, in our experience, is much more likely to be the site of malignant change.

The number of cases of cervical cancer found in these patients seems

surprisingly high (2.08 percent). It must be remembered, however, that biopsy and cytologic, as well as clinical, examination were used to establish a diagnosis of cancer of the cervix in these patients and that half the cases were at a very early stage (carcinoma *in situ*). The low-economic class of patients dealt with here is inclined to neglect disease until definitely symptomatic, which makes the cancer findings in this group more representative of total prevalence rather than incidence of the disease. There are no adequate data available for comparison with the findings here, since prevalence data for the general population are based on known living cases in a population, most of whom have been diagnosed as the result of suspicious symptoms. Our data indicate that there is a considerable reservoir of unknown cervical cancer in a population which can be demonstrated by the means used here.

Comparison of the diagnostic procedures used during this study has been made. For simplicity all clinical procedures such as history and physical examination are grouped as clinical diagnosis, and the two primary laboratory tests are treated separately as the biopsy results and cytologic-test results. This comparison in accuracy is presented in table 2.

The value of biopsies in the diagnosis of early disease is well demonstrated. After the first few months of the study it became very apparent that even the most normal looking cervix clinically may have "carcinoma *in situ*." It then became routine to do a punch biopsy, as well as a cytologic smear, on every cervix presenting no more than minimal surface changes. Small punch biopsies were taken from likely sites, using a Gaylor biopsy instrument, which proved most satisfactory for routine clinical use among several varieties tried. Slight to moderate bleeding was the only sequela encountered after the biopsy procedure, and in every case this was satisfactorily controlled by a tampon placed in the vagina. The biopsy procedure is simple, requires no anesthesia, the use of only simple tools, and can be recommended as a practical office procedure.

The frequency with which the biopsy was employed varied among the several clinicians seeing the cases, but as each examiner had his faith shaken in his clinical ability to detect an early cervical malignancy he tended to employ the biopsy with greater frequency. Thus it developed that the majority of the patients received a cervical biopsy at the time of the initial examination. Despite this only 71.9 percent of the intraepithelial cervical malignancies would have been diagnosed by relying on the liberal use of the biopsy technique based on clinical indication (table 2). In the light of the experience presented, it is obvious that even with use of the cervical biopsy on slight clinical indication some early cervical carcinomas will be missed. Some of these cases present an entirely normal-appearing cervix grossly.

It is not our intent in this discussion to criticize or give the final evaluation concerning diagnostic methods; however, we have certain comments worthy of mention from our own experience with the cytologic technique, which is claiming increasing interest as a case finding and diagnostic

procedure. The efficiency of this test used in our study may be considered as average for moderately well-trained people. There were 2 physicians who had been trained in cytologic-smear diagnosis for several months. Preliminary screening of the slides was done by 2 technicians specifically trained to evaluate cytologic material. In the early days of our study, methods of collection of material for the cytologic test were crude and limited to collections with a cotton swab. Many errors were undoubtedly made because of inexperience and inadequate material; however, when the Ayre spatula (*10*) was first introduced as the means of collection of material directly from the cervix, the efficiency of the cytologic test increased. Eleven of the total 15 cases with an initial false-negative cytologic test were reported prior to the institution of the Ayre-spatula technique. Approximately one-third of the total of 3,224 cases was seen during this period.

The failure of the cytologic test to detect cancer cells in cervical smears from 6 of the 19 advanced cervical carcinoma cases does not represent a serious failure of the test. In all but 1 false-negative case only necrotic, bloody debris was obtained by smear. The cells obtained presented little nuclear detail, and in fairness to the cytologists it should be stated that they scarcely merited interpretation. These cases clinically were obviously advanced cervical carcinomas and the cytologic test was not necessary for diagnosis. The clinicians had difficulty in obtaining even the inadequate cytologic smears due to discomfort to the patient as well as profuse serosanguineous discharge from the cervix.

Other factors than those mentioned above should be considered in the relatively low accuracy of the cytologic test in the early invasive and clinically obvious carcinomas of the cervix. Most important of these are: 1) autolysis of cells due to surface infection; 2) the inclusion on the smears of a large amount of confusing inflammatory cells, cellular debris, bacteria, and other organisms associated with poor hygiene and cervical infections; 3) inadequate material on many slides which often could not be repeated because of the short period patients were domiciled in the diagnostic center and their subsequent departure to widely scattered areas. Even with the above limitations the cytologic test still proved to be of definite value, particularly in the cases of carcinoma *in situ*. Some of these cases would have been missed, histologically, if a positive cytologic test had not made repeated biopsies imperative (table 2).

A total of 814 repeat examinations were performed on 643 of the 3,224 patients examined. Over half of these 643 cases were completely reexamined by the Cancer Detection Clinic at the time of their return for venereal disease follow-up study. The remainder were recalled by the Cancer Detection Clinic because of clinical cytologic, or histologic, suspicion of cancer at the time of the initial or previous examination. In no case did subsequent examination reveal evidence of carcinoma of the cervix that was not indicated by either the biopsy or cytologic test at the time of the initial examination. Under the conditions of this study the combined accuracy of the 2 techniques in diagnosing carcinoma of

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the cervix approaches 100 percent. Graham, Sturgis, and McGraw (*11*) reported 181 cases of cervical cancer with failure of either a biopsy or cytologic test to diagnose the condition at the time of the first examination in only 3 instances, for a combined accuracy of 98.3 percent.

One other point should be made which is neglected in this study. Only cases finally proved by histopathology have been included here. A sizable number with suspicious or positive cytology were not proved to have cancer from biopsies taken during their initial observation. Follow-up examinations of most of these had not been completed at the time the authors left this project. Some of these will probably be found to have cancer, and this would change some of the data presented in this report.

SUMMARY

A series of 3,224 female patients studied in a cancer detection clinic has been presented. Sixty-seven primary carcinomas of the cervix were found in these patients. The methods used in the detection and diagnosis of these cases, including clinical, pathologic, and cytologic, have been compared and evaluated. The pitfalls and stumbling blocks in the early detection of carcinoma of the cervix have been cited. Particular study has been made of 32 cases of carcinoma *in situ* and an evaluation of the symptoms, clinical findings, histologic studies, and cytologic-smear tests has been presented.

CONCLUSIONS

1. Carcinoma of the cervix in all stages of development is a prevalent disease (2.08 percent in this report) in the colored female population of the South in the cancer age and with venereal disease.

2. The data presented suggest that the majority of cervical malignancies existing in a group of female patients are present in an asymptomatic, localized form for a minimum of 5 to 7 years before the typical symptoms and clinical appearance of cancer become apparent.

3. It is possible to achieve a high degree of accuracy in diagnosing early unsuspected cervical carcinoma by the combined use of the cytologic test for cancer and the tissue biopsy.

4. Even a detailed history is of little practical value in diagnosing early cervical malignancies.

5. The ability of a clinical examiner to discriminate between early cervical malignancies and benign conditions is grossly inaccurate.

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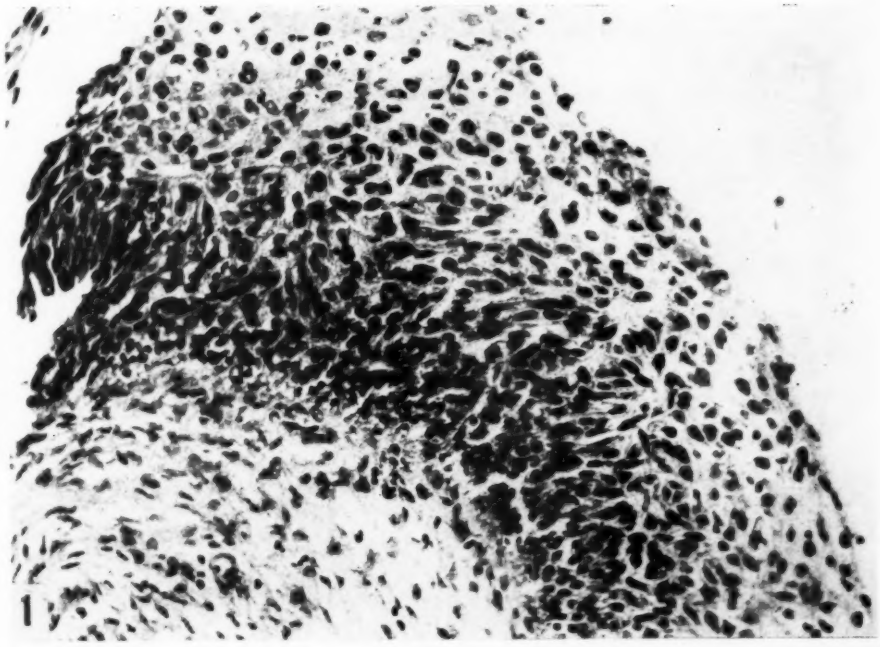
FIGURES 1 and 2.—Carcinoma *in situ* of cervix. Hematoxylin and eosin stain. × 340.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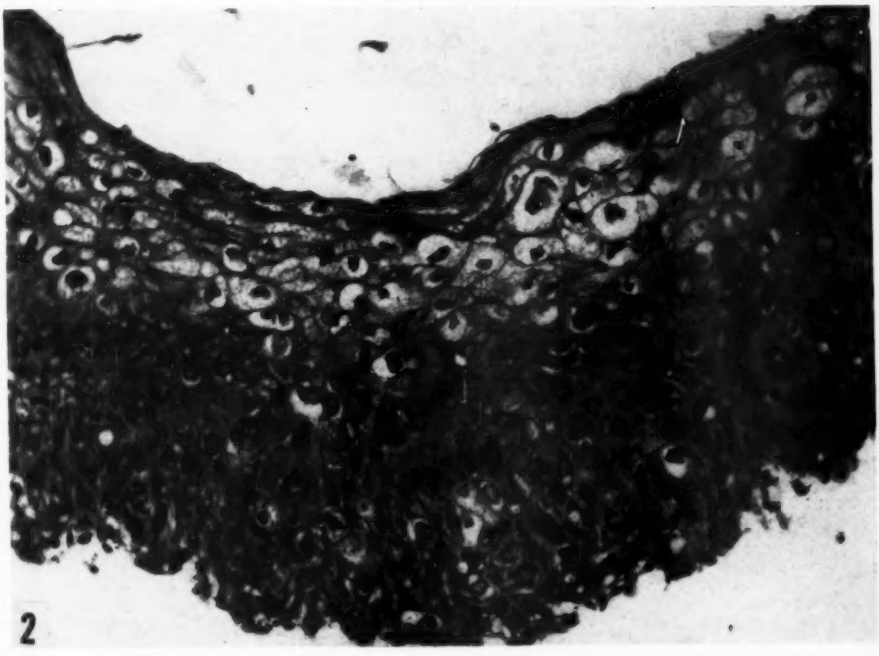
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THE DISAPPEARANCE OF SULFHEMOGLOBIN IN THE BLOOD AS A MEASURE OF THE LIFE SPAN OF ERYTHROCYTES IN NORMAL RABBITS AND MICE AND IN TUMOR-BEARING MICE[1,2]

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The currently accepted methods of measuring the life span of erythrocytes involve the use of isotopic nitrogen, as described by Shemin and Rittenberg (*1*), of radioactive iron, as reported by Cruz, Hahn, and Bale (*2*), or by use of the differential agglutination method of Ashby (3).

Jope (*4*) has shown that sulfhemoglobin pigment disappears slowly from the blood of patients with clinical sulfhemoglobinemia. The disappearance of sulfhemoglobin reflects the rate of destruction of erythrocytes, since sulfhemoglobin is released and excreted only after dissolution of the erythrocyte. Thus, the disappearance of sulfhemoglobin from the blood may be used as a measure of the intravascular life span of erythrocytes, if the cell is not injured and its life span is not affected by the formation of sulfhemoglobin. McKerns and Denstedt (*5*) applied this method in rabbits. When sulfhemoglobinemia was developed *in vivo* in these rabbits by the administration of phenacetin and sulfur, the rate of disappearance of sulfhemoglobin indicated an average erythrocyte life span of 20 days. When such blood was transfused into other rabbits, the red-blood-cell life was found to be 10–12 days. With the development of sulfhemoglobin *in vitro* by bubbling hydrogen sulfide gas through oxygenated blood, and then infusing the blood into rabbits, the maximum life span of the erythrocytes was 8 days. The results of McKerns and Denstedt demonstrated that the possible use of the sulfhemoglobin-tagging of red blood cells as a method of measuring their life span required the development of a simple, rapid, and safe means by which sulfhemoglobin *in vivo* could be produced.

Clinical sulfhemoglobinemia has been described following exposure to phenacetin, acetanilid, sulfonamides, and trinitrotoluene, if at the same

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time sulfur is ingested or is present in the gastroenteric tract (*4,6,7*). Sulfanilamide administered to mice will cause methemoglobinemia; if sulfur, particularly as sodium sulfide, is added to the diet, sulfhemoglobin is formed (*8*). Para-aminopropiophenone is an active former of methemoglobin (*9*), and it was decided to study the ability of this agent to form sulfhemoglobinemia *in vivo*.

The purpose of the present investigation was to tag erythrocytes with sulfhemoglobin for the measurement of their life span. This entailed the development of sulfhemoglobinemia *in vivo* in rabbits, and the application of this method to the study of the life span of erythrocytes in rabbits, normal mice, and mice bearing transplanted mammary carcinoma.

EXPERIMENTAL PROCEDURE

Determination of Sulfhemoglobin in Blood

The method used for determining the level of sulfhemoglobin pigment in the blood was essentially the same as described by Jope (*4*). Density readings were made at wave lengths 540 and 620 mμ. All measurements were made using a Beckman Quartz Spectrophotometer, Model DU, with cells 1 cm. in thickness. The dilution fluid was 0.4 percent ammonia, which was adequate to clear the solution and to prevent changes in pH. No marked effect on the density was observed when density readings were taken at 620 mμ of various solutions from pH 6.6 to 10.6.

The density of blood containing 0.5 to 10 percent sulfhemoglobin and diluted 1:100 varied from 0.02 to 0.120 at 620 mμ. At these low densities duplication of results was difficult. However, when the dilution was 1:10 the density ranged from 0.200 to 1.20 and the results were much more consistent. Therefore, this latter dilution was used whenever possible for the determination at 620 mμ. The density at 540 mμ was too high in a 1:10 dilution, so that at this wave length 1:100 dilutions were used.

The ratio

$$\frac{\text{D620} \quad \text{1:10 dilution}}{\text{D540} \quad \text{1:100 dilution}}$$

gave values that could be followed to a baseline determined on untreated animals. This baseline was quite standard in normal untreated rabbits and mice, since it was essentially the ratio of the extinction coefficient of oxyhemoglobin at these wave lengths. It was impossible to obtain enough blood from mice for the 1:10 dilution and, therefore, the ratio

$$\frac{\text{D620} \quad \text{1:100 dilution}}{\text{D540} \quad \text{1:100 dilution}}$$

was used in these animals. These ratios are proportional to the amount of sulfhemoglobin in percent.

The life span of the erythrocyte was taken as that time for the ratio $\frac{\text{D620}}{\text{D540}}$ to return to the initial value, or for all sulfhemoglobin to disappear. The ratio $\frac{\text{D620}}{\text{D540}}$ was plotted logarithmically as a function of time.

Production of Sulfhemoglobin in Vitro

Hydrogen sulfide gas at 10 cc. per minute for 20 minutes was bubbled through oxygenated rabbit blood. The red blood cells were washed in 5 percent dextrose in water, and injected into a second rabbit. An adequate sulfhemoglobin level was obtained in the recipient, but the pigment disappeared in 3 days. It was concluded that the cells had been damaged by this treatment, and this line of investigations was abandoned in favor of producing sulfhemoglobin *in vivo*.

Production of Sulfhemoglobinemia in Rabbits

Normal market rabbits, weighing 2 to 5 kg., were given various drugs to determine optimal combinations of drugs and routes of administration in order to develop sulfhemoglobinemia. Phenacetin, acetanilid, sodium nitrite, and para-aminopropiophenone were used as oxidizing drugs, and sulfur, calcium sulfide, and sodium sulfide as sulfur-containing drugs. The doses of these chemicals, the routes of administration, and the levels of sulfhemoglobin obtained are recorded in table 1.

TABLE 1.—*Production and disappearance of sulfhemoglobinemia in rabbits*

Experiment	Weight, kg.	Oxidizing drug	Amount given and route [1]	Sulfur-containing drugs	Amount given and route [1]	Days to reach sulfhemoglobin level	Approximate percent sulfhemoglobin developed	Red-cell life (days)	Remarks
1	5.0	Phenacetin	0.6 gm. daily PO	Sulfur	1.0 gm. daily PO	29	5	32.5	Straight line.
2	4.0	Acetanilid	0.04 gm. daily PO.	do	do	22	23	41.0	Do.
3	4.5	Para-aminopropiophenone.	5, 10, 10 mg. on 3 successive days IM.	Calcium sulfide.	100, 200, 200 mg. on 3 successive days IM.	3	25	57.0	Do.
4	4.0	do	65 mg. PO	Sulfur	1.0 gm. daily for 12 days before injection PO.	1	27	46.0	Do.
5	4.0	do	50 mg. IP	Sodium sulfide.	120 mg. slowly IV	[2]	16	[3] 27.0 [4] 47.0 [5] 35.0	2 slopes, steep at first, then leveled off.
6	4.0	Acetanilid	0.4 gm. daily PO	Sulfur	1.0 gm. daily PO	7	11	41.0	Slope changes. Curve wavy suggesting rate change.
7	4.0	Para-aminopropiophenone.	20 mg. IM	Calcium sulfide.	400 mg. IM	1	18	43.0	Straight line.
8	3.0	Sodium nitrate	0.4 gm. daily IV.	Sulfur	1.0 gm. daily PO	12	2	26.0	Straight line. Developed marked anemia with regeneration during period measured.
9	4.5	Para-aminopropiophenone.	60.0 mg. IP	do	1.0 gm. daily for 10 days before injection PO.	1	65	9.0	Straight line. Rapid anemia, with rapid regeneration.
10	2.0	Acetanilid	0.4 gm. daily PO.	do	1.0 gm. daily PO	7	5	18.0	Slight reverse S curve. Anemia with rapid regeneration.

[1] PO—oral, IM—intramuscular, IP—intraperitoneal.
[2] Immediate. [3] Initial. [4] Final. [5] Average.

Oral sulfur alone, given in doses of 1.0 gm. daily for 14 days in rabbits, produced a level of sulfhemoglobinemia of 3 percent. Sodium nitrite intravenously with sulfur orally produced no greater level of sulfhemoglobin than sulfur alone. Phenacetin and acetanilid, with sulfur, resulted in sulfhemoglobinemia of 5 to 25 percent.

Levels of sulfhemoglobin of 10 to 20 percent were obtained in 24 hours in rabbits by intramuscular administration of para-aminopropiophenone mixed with calcium sulfide. If sulfur was given orally for 4 to 5 days previously, a level of sulfhemoglobinemia of 10 to 20 percent was obtained within 1 day either by oral or parenteral use of para-aminopropiophenone. With this procedure, there was no evidence of hematopoietic damage with sulfhemoglobin blood levels of up to 25 percent, as revealed by a stable hemoglobin level.

Production of Sulfhemoglobinemia in Mice

On the basis of the experience in rabbits, para-aminopropiophenone and calcium sulfide, suspended in 0.1 cc. of sesame oil, was injected subcutaneously to induce sulfhemoglobinemia in mice (table 2). This

Table 2.—*Disappearance of sulfhemoglobinemia in C3H mice—normal and with mammary tumors—following subcutaneous administration of para-aminopropiophenone and calcium sulfide*

C3H mice	Age (months)	Para-aminopropiophenone, mg.	Calcium sulfide mg.	Approximate percent sulfhemoglobin	Erythrocyte life (days)	Type of curve
Series I. Normal	4					
No. 1		0.25	5	4	18.5	Straight.
No. 2		.50	10	6	19.5	Do.
No. 3		.75	15	11	21.0	Do.
No. 4		1.00	20	7	19.5	Do.
No. 5		1.50	30	17	19.5	Do.
Series II. Normal	3					
No. 6		1.00	20	9	20.5	Do.
No. 7		1.00	20	9	22.0	Do.
No. 8		1.00	20	8	18.5	Do.
Series III. Normal	3					
No. 9		1.00	20	7	19.0	Do.
No. 10		1.00	20	15	17.5	Do.
No. 11		1.00	20	13	17.5	Do.
No. 12		1.00	20	12	20.0	Do.
No. 13		1.00	20	10	18.0	Do.
No. 14		1.00	20	9	18.0	Do.
Series I. Mammary tumors.	3					
No. 15	(1)	1.00	20	9	10.5	Increasing slope.
No. 16	(1)	1.00	20	7	10.5	Do.
No. 17	(1)	1.00	20	7	11.0	Do.
No. 18	(1)	1.00	20	10	9.5	Do.
No. 19	(1)	1.00	20	9	10.5	Do.
No. 20	(1)	1.00	20	10	10.0	Do.
Series II. Mammary tumors.	3					
No. 21	(1)	1.00	20	17	7.0	Do.
No. 22	(1)	1.00	20	19	7.0	Do.
No. 23	(1)	1.00	20	13	7.0	Do.
No. 24	(1)	1.00	20	20	7.0	Do.

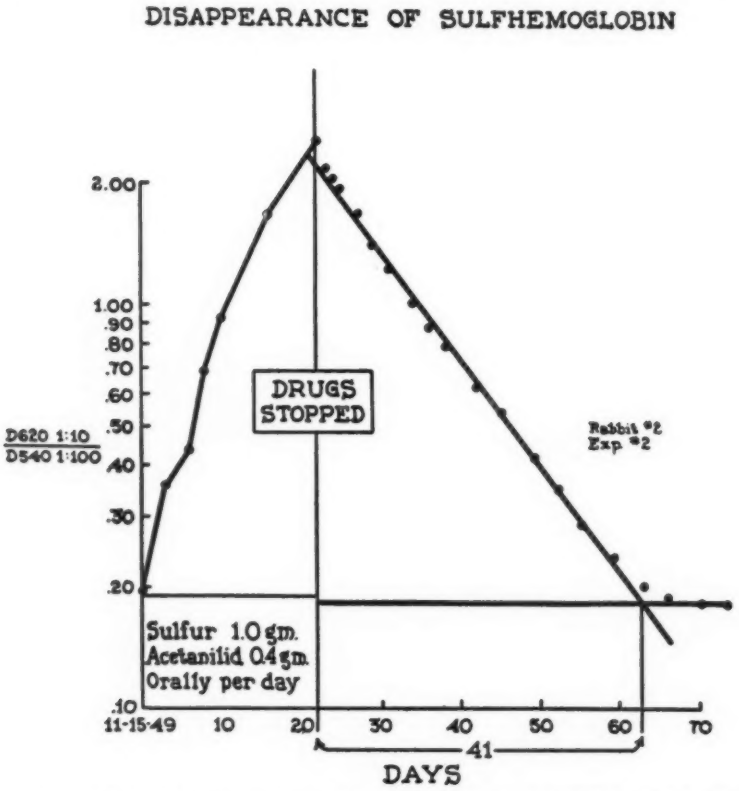
[1] 7 days after implant of tumor.

method afforded adequate levels of sulfhemoglobinemia, and the best curves of disappearance of sulfhemoglobin from the blood.

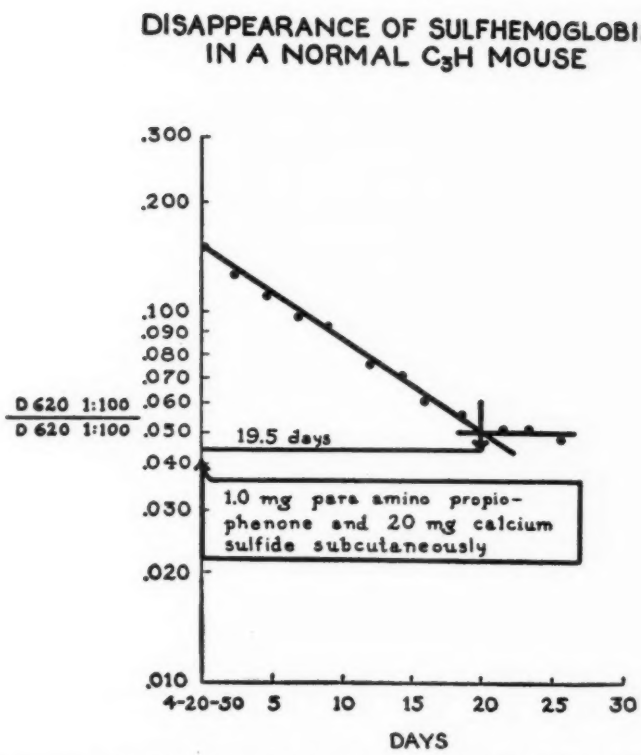
Serial determinations of sulfhemoglobinemia were made on 24 male mice of the C3H strain. The animals were approximately 3 months old and weighed 25 to 30 gm. A mammary adenocarcinoma, which originally arose in a C3H female, was transplanted in 10 of these mice. The tumors were implanted subcutaneously, and the sulfhemoglobinemia was induced in the animals 7 days later, when the tumors measured approximately 0.5 cm. in diameter.

RESULTS AND DISCUSSION

The ratio $\frac{D620}{D540}$, when plotted logarithmically as a function of time, resulted in a straight line in 7 normal rabbits and in the 14 normal mice used in the study (text-figs. 1 and 2). This finding does not agree with the



TEXT-FIGURE 1.—The disappearance of sulfhemoglobin in a rabbit in which sulfhemoglobinemia of 25 percent was induced by sulfur and acetanilid orally. Note the straight-line relationship between the logarithmic ratio $\frac{D620}{D540}$ plotted against time. Complete disappearance of sulfhemoglobin, and presumably the tagged red blood cells, occurred in 41 days.



TEXT-FIGURE 2.—Disappearance curve of sulfhemoglobin in a normal mouse.

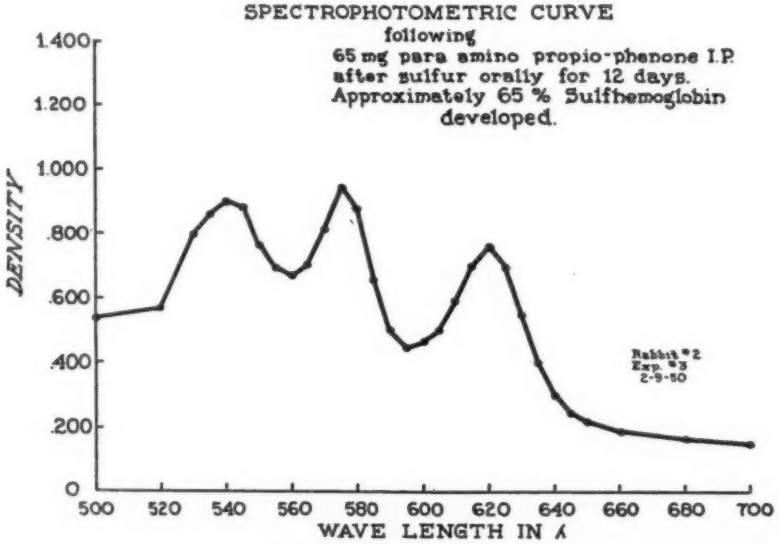
work of Jope (*4*), who found a linear relationship between the amount of sulfhemoglobin and elapsed time, both plotted on an absolute scale. The data would indicate that sulfhemoglobin is formed equally in the red-blood-cell population, and that the subsequent destruction of the erythrocytes is at random. It is not established that sulfhemoglobinemia does not alter the life span or the destruction rate of such cells, although sulfhemoglobinemia up to 25 percent did not result in clinical or hematological evidence of damage to hematopoiesis, as indicated by a stable hemoglobin level during the test period. McKerns and Denstedt (*5*) showed that the red blood cells can be sulfurated up to a level of 50 percent without seriously altering their normal osmotic stability (fragility) and without impairing the agglutinability with Anti-A, -B, -M, or -N serum. Jope (*4*) found that 7 percent of the hemoglobin of the cells can be converted to sulfhemoglobin in the human without affecting the life span of the red cell. McKerns and Denstedt (*5*) also showed that the results of tests for estimation of blood volume agreed well with those by the dye method and that the partial damage to viability caused by the *in vitro* conversion of sulfuration did not introduce a serious error.

For final evaluation in regard to the reliability of this method for deter-

mining life span of erythrocytes, the procedure requires comparison with such currently accepted techniques for the measurement of erythrocyte life span, as the isotopic nitrogen or iron determinations.

The average period for the disappearance of sulfhemoglobin in 7 rabbits was 43 days, with a range of 33 to 57 days. This value for the life span of erythrocytes in rabbits agrees closely with the results of Kurtz (*10*)—using the method of recurrent reticulocyte showers—who obtained an average of 42 days, with a variance of 16 to 73 days.

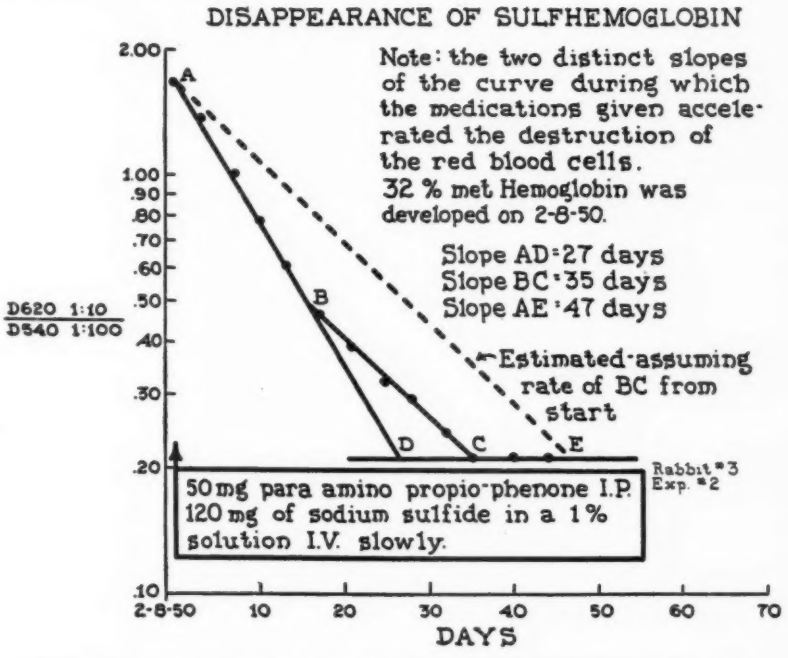
In three rabbits, a marked anemia was noted after the formation of sulfhemoglobinemia. One rabbit was given sodium nitrite intravenously and sulfur orally (table 1). A low level of sulfhemoglobinemia appeared and was attributed to the sulfur alone, since rabbits fed only sulfur developed comparable levels of sulfhemoglobin. The rabbit subsequently developed a marked hemorrhagic tendency and a severe anemia. The sulfhemoglobin disappeared from the blood in 26 days, and regeneration of erythrocytes occurred as indicated by an increase in the total hemoglobin. Anemia also occurred in the second rabbit, given acetanilid and sulfur orally. An increase in hemoglobin was observed as the sulfhemoglobin disappeared during the next 18 days. In the third rabbit, oral sulfur and parenteral para-aminopropiophenone was given and approximately 65 percent of the hemoglobin was converted to sulfhemoglobin (text-fig. 3). A profound anemia developed and regeneration was accompanied by a 25 percent reticulocytosis. The sulfhemoglobin pigment was



TEXT-FIGURE 3.—Spectral curve of blood from a rabbit with 65 percent sulfhemoglobinemia induced by para-aminopropiophenone given intramuscularly after oral feeding of sulfur. Measurements in 1:100 dilutions of 0.4 percent ammonium hydroxide in water.

cleared from the blood in 9 days. Thus, in 3 rabbits with evidence of marked hematopoietic disturbance the sulfhemoglobin disappeared in 9, 18, and 26 days.

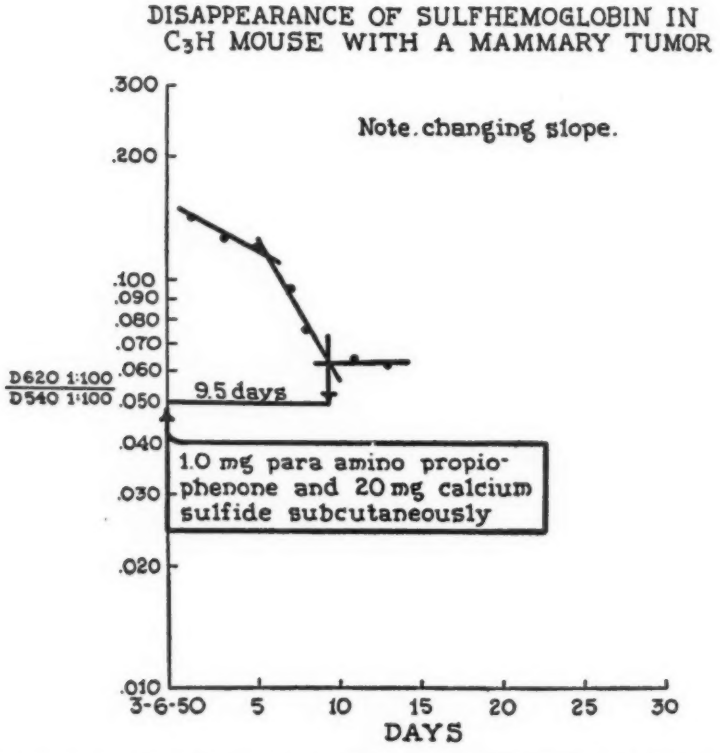
One of the objectives of this study was to develop a quick level of sulfhemoglobin without disturbing the hematopoietic system. In Experiment No. 5 para-aminopropiophenone was given intraperitoneally and a 30-percent-methemoglobin level resulted. One-percent-sodium-sulfide solution was then given intravenously at a sublethal rate. The next day a 15-percent-sulfhemoglobin level was attained, with a typical spectral curve. The decay curve was rapid at first but later slowed. This indicated that some change occurred during this time to alter the life of the red blood cells (text-fig. 4).



TEXT-FIGURE 4.—Changing slope of sulfhemoglobin disappearance in a rabbit.

In the 14 normal C3H male mice, the average life span of erythrocytes, as measured by the disappearance of sulfhemoglobin from the blood, was 19.2 days, with a range from 17.5 to 21 days (table 2). In 10 mice with transplanted mammary carcinoma, the average life span of erythrocytes was 9 days, with a range of 7 to 11 days (table 2). This difference is statistically significant. All 10 determinations in C3H mice with mammary tumors showed a changing slope when plotted as a logarithmic function (text-fig. 5). A profound anemia developed as the tumor progressed, and simultaneously the sulfhemoglobin level decreased more

rapidly. Consequently, the mice with tumors showed an increasing slope of their curve of sulfhemoglobin disappearance as they approached death (text-fig. 5).



TEXT-FIGURE 5.—Changing curve of sulfhemoglobin disappearance with the growth of a transplantable mammary carcinoma in a C3H mouse. A red-cell life based on the initial slope is 20 days approximately that found for normal mice, while the red-cell life indicated by the second slope gives a red-cell life of 9.5 days.

CONCLUSIONS

1. Sulfhemoglobinemia was induced in rabbits and mice, particularly by the parenteral use of para-aminopropiophenone in combination with sulfide ion.

2. The disappearance of sulfhemoglobin pigment from the blood, plotted logarithmically as a function of time, elicited a straight-line relationship in normal rabbits and mice. The slope was accelerated in the presence of red-blood-cell destruction or transplanted tumor.

3. The sulfhemoglobin disappearance, and presumably the life span of the erythrocytes, occurred in an average of 43 days (32.5 to 57) in 7 rabbits.

4. In three rabbits in which anemia was induced during the develop-

ment of sulfhemoglobin, the rate of disappearance of sulfhemoglobin was increased to 9, 18, and 26 days.

5. In 14 normal C3H male mice, the sulfhemoglobin disappeared in an average of 19.2 days (17.5 to 21).

6. In 10 C3H mice bearing transplanted mammary carcinoma, the sulfhemoglobin disappeared in 9 days (7 to 11 days).

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HEREDITARY AND PATHOGENIC NATURE OF MUTANT MITOCHONDRIA IN *NEPETA* [1] [2]

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INTRODUCTION

The dominant role of the mitochondria in cellular metabolism is becoming apparent from studies involving both plant and animal cells [*4, 10, 11, 12, 17*; see Bradfield (*3*)]. The hereditary system of mitochondria, the chondriogenes (*7*), in a special case called plastogene by Imai (*15*), has been investigated chiefly in higher plants where mutant mitochondria differentiate into plastids with characteristic abnormalities. Because of this fact it has been possible to obtain considerable information concerning the hereditary structure of mitochondria. It is known that mitochondria in mutant states can become the continuing cause of neoplasia characterized by distinctive changes in cellular enzymic activities (*8, 30*). The extent to which the mitochondria of a single species may undergo mutation, and the possible influence of such aberrant mitochondria on cellular metabolism, growth, and differentiation, are basic considerations in evaluating the potential role of mutant mitochondria in the causation of neoplasia. The present paper reports the results of such a study in *Nepeta cataria*, L.

In 1946 Dr. E. A. Walker [4] of the United States Department of Agriculture found a single variegated plant of *Nepeta cataria*, L. (catnip) from which it has been possible to obtain an extraordinarily high number of mutant mitochondrial types. These types, described in the first part of this paper, range from those producing variegational diseases of very mild character to those symptomatically indistinguishable from highly pathologic viroses of the rugose mosaic type. That these abnormal types of mitochondria or their derivatives are the result of multiple mutations in the chondriogenes of this clone or its progeny will be shown in the second

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[2] Acknowledgment is made to the Maryland Agricultural Experiment Station, Department of Botany, University of Maryland, where this study was begun. Facilities supplied by the University of Maryland and the National Institutes of Health allowed completion of the study.

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[4] The authors acknowledge the valuable contribution of Dr. E. A. Walker, whose discovery of the original mutable plant of *Nepeta* made this investigation possible.

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part of this paper. Also, the occurrence of these mitochondrial mutations will be shown to be associated with a recessive nuclear gene. This gene, designated *m*, although not indispensable for chondriogene mutation, is essential for a high rate of mutation to occur. The gene is not at all necessary for the continued propagation of mutant mitochondrial types once these have appeared. Since the mode of action of this gene has not been determined, it will be referred to merely as the mutation-allowing gene rather than by the more committal term of mutagenic gene. The data have a bearing on the mitochondrial theory of virus origins and on the hereditary structure of mitochondria.

HISTORICAL

Yasui (*32*) reported two types of mutant plastids in *Hosta* which differed in the amount of pigment contained in them. She was able to demonstrate seven cell types corresponding to the possibilities for segregation of three types of plastids. Imai (*13*) described tricolored plants of *Hordeum* having normal, yellow, and white plastids although cytological details were not given. Imai (*14*) was also able to show that nuclear genes can stimulate the mutation of normal plastids. Rhoades (*21*) reported a recessive nuclear gene in *Zea* which was associated with the appearance of albino plastids. The perpetuation of such aberrant plastids was not dependent upon the continued presence of this gene. Sô (*25*) and Pal and Ramanujam (*19*) have reported a similar mutagenic nuclear mechanism. In all these cases the extent of plastid mutation was limited to a few types of variants, none of which produced pronounced pathologic changes in cell growth and differentiation.

Previous studies (*6*, *29*) have shown that certain leaf variegations, which are caused by abnormal mitochondria, can be arranged on the basis of symptoms into a series showing progressive similarity to typical virus diseases. Reductions in size and structural complexity of the mutant plastids were proportional to the increasing similarity to a virose-like syndrome. These observations were of necessity based on mitochondrial types of different species and genera because most plants show very low rates of "spontaneous" mutation of their chondriogenes. Estimates of the capacity of the chondriome of any *one* species to vary were therefore largely conjectural.

TERMINOLOGY

In order to distinguish between abnormal plastid types which have the property of self-perpetuation independent of the genomic constitution of the plant, and those plastid abnormalities which, on crossing, occur according to Mendelian genetics, the term *chondriogene* is used in order to avoid the term "entity of the mitochondria and their derivatives, responsible for the self-perpetuation of one or more traits, independent of the nuclear constitution of the cell in which the mitochondria or their derivatives occur." This term is contrasted with the term gene or nuclear gene, denoting the entity of the nucleus affecting the characteristics of a cell [see duBuy and Woods (*7*) for justification of preference of chondriogene

over plastogene]. Derived from this term are: *chondriogene mutation*, indicating a newly-occurring, self-perpetuating change in mitochondria or their derivatives, as contrasted to genic mutation, a change occurring as a result of nuclear action; *homochondric*, denoting that the mitochondria or their derivatives within one cell are of *one* self-perpetuating type, as contrasted to homozygotic; *heterochondric*, indicating that the mitochondria and their derivatives derive from two (dichondric) or more (polychondric) independently self-perpetuating types, as contrasted to heterozygotic; and *chondriogenotype*, a sum total of the entities responsible for the self-perpetuation of a certain type of mitochondria or their derivatives, as contrasted to genotype or nuclear genotype. A *mutant chondriogenotype* will represent a sudden change in the chondriogenotype, which is self-perpetuated and is sexually inherited in non-Mendelian fashion.

MATERIALS AND METHODS

Plants were grown in washed sand or in vermiculite, and nutrients supplied as a commercial water-soluble fertilizer or from reagent chemicals.[5] The plants flowered freely during the summer but required supplemental illumination during the winter. Cytological studies were based on living tissues because neutral formalin or other fixatives frequently caused serious alterations in cell structures. Leaf tissues were placed in the nutrient salt solution with 5 to 10 percent sucrose added. Air was rapidly withdrawn from the immersed tissues with a high-vacuum pump and the vacuum suddenly released. This was repeated until the intercellular spaces were filled with solution. Sections were cut immediately with a sharp razor and mounted in the same solution. Active cyclosis could be maintained in mesophyll cells by causing the solution to flow slowly under the cover glass during observation. Material mounted in an Aston flow cell (*28*) could be kept alive for a day or more and was used to advantage in vital staining. All photomicrographs or drawings are of living cells.

Emasculations and pollinations were readily accomplished with the use of a 4 × binocular magnifier. Flowers were bagged to protect them from chance pollination. Seeds were germinated in sterile sand or vermiculite. Standard techniques for mechanical transmission of viruses were employed together with grafting procedures to detect any variegation-inducing viruses that may have been present in the experimental material. All such tests were negative. Studies of the number and behavior of meiotic chromosomes were made in freshly prepared aceto-carmine smears of anthers.

EXPERIMENTAL RESULTS

I. Cytological

A. *Frequency of occurrence of abnormal mitochondria or their derivatives.* Field surveys of natural stands of *Nepeta cataria*, L., failed to reveal any

[5] The commercial fertilizer was obtained from the Miller Chemical Co. of Baltimore, Maryland, under the trade name of VHPF. The prepared nutrient solution had the following composition (millimolar concentrations): NH_4NO_3, 3.56; KH_2PO_4, 2.56; $CaSO_4$, 4.80; $MgSO_4.7H_2O$, 17.0; $MnSO_4.2H_2O$, 0.035; $ZnSO_4.7H_2O$, 0.0066; H_3BO_3, 0.042; Ferric tartrate, 0.0046.

variegated individuals although several thousand plants were examined. In one location, however, plants infected with a graft-transmissible chlorosis were found. These are discussed later.

The behavior of a mutable clone of *Nepeta cataria*, L., was in marked contrast to the "stable" green clone, which was established from one of the "wild" catnips. A large number of different mitochondrial types occurred among its offspring and, as is shown in the second part of this paper, their occurrence was under the influence of a mutation-allowing gene, *m*. These mutant mitochondria or their derivatives could be characterized by their effect on the growth and differentiation of the cells containing them, and by abnormalities in the plastids that derived from these mutant mitochondria. These plastid abnormalities included changes in size and pigment content, changes in internal structure, and changes in enzymic activities. Table 1 and the figures present the characteristics of 15 different mutants that have appeared in our material during the last 4 years. The total number actually observed is considerably greater, but all of the types have not been adequately characterized. It is almost certain that some distinct though closely similar forms have not been differentiated from each other. To characterize a given chondriogenotype adequately, it is necessary to study its behavior free from admixture with other abnormal types. This often requires considerable asexual or sexual propagation to achieve the necessary segregation of mitochondria. The range of variation extends from mitochondrial mutants that are capable of developing into only slightly abnormal plastids through intermediate forms that differentiate into strongly modified plastids, to mitochondrial types apparently totally incapable of developing into plastids (figs. 1–9). Certain derivatives, however, are apparently no longer able to function as an independent chondriome. These have a pathogenic action similar to that produced by infectious viruses of the rugose mosaic type in other plants. However, none of these agents arising in *Nepeta* have been transmissible in grafting or mechanical inoculation tests (table 2).

TABLE 2.—*Summary of the attempts to demonstrate, by graft transmission, virus-like agents in plants of* Nepeta cataria, *L., containing mutant mitochondria or their derivatives. Good graft unions were obtained in all cases. No evidence of transmission to normal plants was obtained*

GENOTYPES[1]		Number of plant pairs	Predominant type of abnormal mitochondrial derivatives	Days since grafting (average)
Plant with normal mitochondria	Plant with abnormal mitochondrial derivatives			
MM	*mm*	18	Variegation-inducing types	106
Mm	*mm*	8	do	52
Mm	*Mm*	1	do	78
Mm	*Mm*	2	Growth-distorting factors[2]	62
MM	*Mm*	6	Variegation-inducing types	82
MM	*Mm*	4	Growth-distorting factors[2]	82

[1] *M* represents dominant gene which completely eliminates mutation-allowing influence of recessive *m*.
[2] 2 of these were of the 35–23 type and 4 of the 34–2 type. Many additional attempts to graft-transmit the 35–23 type were also negative.

TABLE 1.—*Characteristics of certain chondriogene mutants*

Chondriogene type	Approximate size ratio[1]	Effect on plastid differentiation				Effect on morphology of nonnormal plastids in heteroplastic cells	Effect on cell division	Effect on cell and diffe
		Color		Grana structure	Structural changes as cell matures			
		Young cells	Old cells					
Normal	1.0	Normal green	Normal green	Small (ca.0.5μ) 200± per plastid.	Form maintained			
Supernormal type 1	.78	Darker-than-normal green.	Darker-than-normal green.	Normal type	do	Apparently none	Stimulation	None
Light green type 1	1.4	Medium light green.	Very light green	Approximately normal.	Slight vacuolation	do	None	None
Light green type 2	1.4	Very light green	Yellow	Apparently absent	Moderate vacuolation	do	do	do
Light green type 3	1.7	do	Very light yellow	do	Considerable vacuolation.	do	do	do
Variable yellow-green type 1.	1.5±	Yellow	Very pale yellow to almost normal green.	Nearly normal to absent.	None to extreme vacuolation.	do	do	do
Macrograna type 1	1.5 (young) .9 (old)	Medium light green.	Light green	2 to 3 × normal diameter (ca. 20 per plastid).	Grana breakdown, large unilateral vacuolate forms.	do	do	do
Macrograna type 2	2.3 (young) 2.3 (old)	Very light green	Yellow green	4 to 5 × normal diameter (ca. 1 to 6 per plastid).	Grana breakdown, becomes highly vacuolate.	do	do	do
Green-spotted cream	4.0	Light yellow-cream to greenish.	Very light yellow	Apparently absent	Pronounced vacuolation.	do	do	do
Cream	1.9	Greenish to yellow-cream.	Very pale cream	do	Considerable vacuolation.	do	do	do
White vacuolate type 1.	2.8	Colorless	Colorless	Absent	Extreme vacuolation	Very little to none	do	Slight re
White vacuolate type 2.	3.0 or more (plastids may not develop).	do	do	do	Become highly vacuolate if plastids develop.	Reduced in size and color.	Probably some retardation.	Conside ductio
Mitochondrial white	No plastids				Mitochondrial form maintained.	Not observed	do	do
34–2 growth-distorting factor.	Mitochondria or submitochondria.				Not known	None to pronounced reduction in size and color.	Strong to moderate retardation.	Pron change shape.
35–23 growth-distorting factor.	do				do	None to moderate reduction in size and color.	do	do
"Spider" growth-distorting factor.	do				do	Apparently none	Apparently none	Leaves a altered

[1] Diameter of normal divided by diameter of mutant.

1.—*Characteristics of certain chondriogene mutants*

tructural changes as cell matures	Effect on morphology of nonmutant plastids in heterochondric cells	Effect on cell division	Effect on cell enlargement and differentiation	Effect
rm maintained				
do	Apparently none	Stimulation	None	Not de
ght vacuolation	do	None	None	None
derate vacuolation	do	do	do	do
nsiderable vacuolation.	do	do	do	do
ne to extreme vacuolation.	do	do	do	do
ana breakdown, large unilateral vacuolate forms.	do	do	do	do
ana breakdown, becomes highly vacuolate.	do	do	do	do
onounced vacuolation.	do	do	do	do
nsiderable vacuolation.	do	do	do	do
treme vacuolation	Very little to none	do	Slight reduction	do
rome highly vacuolate if plastids develop.	Reduced in size and color.	Probably some retardation.	Considerable reduction.	Some
itochondrial form maintained.	Not observed	do	do	Sterile
ot known	None to pronounced reduction in size and color.	Strong to moderate retardation.	Pronounced change, size and shape.	do
do	None to moderate reduction in size and color.	do	do	Slight after
do	Apparently none	Apparently none	Leaves and flowers altered.	Appar redu

ment on	Effect on fertility	Transmission through female gametes	Tendency to replace normal mitochondria	Frequency of occurrence
	Not determined	Not determined.	Moderate	Very rare.
	None	Yes	do	Common.
	do	do	do	Do.
	do	do	do	Very common.
	do	do	Strong	Common.
	do	do	Moderate	Moderately common.
	do	do	do	Relatively rare.
	do	do	Complete replacement rare.	Moderately common.
	do	do	Strong	Very common.
on	do	do	do	Moderately common.
re-	Some reduction?	do	Moderate	Rare.
	Sterile	?	Apparently strong	Do.
ed and	do	?	Coexist with normal.	Do.
	Slight fertility after 2 years.	No	do	Do.
wers	Apparently slight reduction.	do	do	Do.

B. *Cytology of heterochondric tissues.* Extensive cytological analyses have shown conclusively that the normal and mutant plastid types occur in the cells in all conceivable combinations. This is particularly marked where well defined variegational patterns (figs. 10–12) and morphologic types (figs. 13–18) occur. Seven cell types could be found in tissues having 3 types of plastids (figs. 13–25) and 15 cell types (some shown in figs. 26–30) in tissues where 4 plastid types occurred. In this connection it should be stressed that accurate cytological analysis of such complicated types of plastid variegation is not possible unless the appearance of all mutant plastid types involved in leaves of varying physiological age is taken into account (figs. 1–9).

1. *Mitochondrial types exerting little or no effect on growth of the cell.* The types having the highest frequency of recurrence fall within this group (tables 1 and 3). On the basis of chlorophyll formation by the mutant plastid, three general subgroups can be recognized.

(*a*) Plastids only slightly changed in morphology (at least during some stage of development) although a noticeable reduction in pigmentation occurs: Examples are "light green type 1" (fig. 31) and "variable yellow-green type 1" (figs. 37–41). The latter is remarkable for the tremendous variability in pigment content, which seems to be determined by both external and internal environmental factors such as, *e. g.*, position in the leaf, nutrition, shading, *etc.*, which would be confusing if they were not studied at various stages of development and obtained from various areas of a plant. Since the observations on this mutant mitochondrial type may have some bearing on the types of variegation in other plants known as "status albomaculatus" [Rhoades, (*22*)], they are presented in some detail. This mutant appeared a number of times and has been transferred to plants with all possible diploid genetic constitutions with respect to the genes *M* and *m*. Cells containing various combinations of normal and mutant plastids produce typical mosaic or sectorial patterns of variegation (fig. 41). The mutant plastids in young leaves are typically yellow (table 1) and vacuolate. As the leaf matures, they often assume a more or less normal form with variable content of chlorophyll. All of these conditions frequently occur in the same leaf with a more or less defined pattern (fig. 38). Lowering the light intensity, together with provision of an abundant supply of mineral nutrients, encourages the formation of chlorophyll in these mutant plastids. The physiological limits for retention of chlorophyll, however, must be very narrow because areas in many leaves become secondarily yellowed and even necrotic (fig. 40). Such secondary loss of pigments is accompanied by extreme vacuolation. It is of interest that leaves containing only the variable yellow-green type of mutant mitochondria and showing the range in symptom expression presented in figure 38, contain single cells in which a considerable range in vacuolation and pigmentation occurs. In other words, the retention of pigment and the vacuolation of these abnormal plastids are not only irregular in different parts of the same leaf but may vary within one cell. It should be stressed that although the mutant

TABLE 3.—*The behavior of mutant mitochondria in Nepeta crosses. The "variegation index" (col. 9) is based on the percentage of the total population showing any degree of variegation, multiplied by the percentage of these variegated plants having more than an estimated 20 percent of the soma mutant. Seedlings with the nuclear constitution mm undergo repeated chondriogene mutation and therefore the mutant mitochondrial types observed probably came in part from this source (entirely so in the case of cross type 6)*

Type cross	Genotypes [1] and chondriogenotypes [2] of parents				Seedlings			Variegation index	Percent of variegated plants tri- or tetrachondric	Mutant mitochondrial types appearing in the seedlings [3] (percent of total number of variegated plants)				
	Female		Male		Number	Probable genotypes	Percent total number variegated			Very light green types	Medium light green types	Cream types	White types	Growth-distorting factors
	Nucleus	Mitochondria	Nucleus	Mitochondria										
1	*MM*	Normal	*MM*	Normal	239	*MM*	0							
2	*MM*	do	*Mm*	do	47	*MM, Mm*	0							
3	*MM*	do	*mm*	Traces of mutants.	105	*Mm*	0							
4	*MM*	do	*Mm*	100 percent white vac. type 1.	6	*MM, Mm*	0							
5	*MM*	do	*mm*	25–95 percent white vac. type 1, macrograna 1, cream.	292	*Mm*	27	0.02	12	49.9	2.6	64.0	5.0	0
6	*Mm*	do	*Mm*	Normal	232	*MM, Mm, mm*	23	[4].05	40	69.2	21.1	50.0	3.8	1.9
7	*mm*	Traces of mutants.	*MM*	do	140	*Mm*	69	.12	24	69.7	6.2	36.4	11.4	4.1
8	*mm*	do	*mm*	Like female	106	*mm*	100	.33	69	79.2	21.6	69.8	9.3	0
9	*mm*	ca. 50 percent white vacuolate 1, macrograna 1, light green 3.	*MM*	Normal	104	*Mm*	81	.41	[5] 26	[5] 70.3	16.3	16.3	16.3	4.8
10	*Mm*	ca. 70 percent cream types.	*MM*	do	126	*MM, Mm*	86	[6].63						
11	*Mm*	50–100 percent white vacuolate 1.	*Mm*	Like female	96	*MM, Mm, mm*	74	.74	[7] 1	0	0	0	100	0

12	*mm*	ca. 50 percent cream (and light green?)	*mm*	-----do------	11	*mm*	100	.91	27	81.8	0	45.5	9.1	0
13	*mm*	75–90 percent various mutants.	*mm*	Traces of mutants.	49	*mm*	100	1.00	------	------	------	------	------	------

[1] *M* represents dominant gene which completely eliminates mutation-allowing influence of recessive *m*.
[2] As judged by mutant plastid types present. Traces of mutants refer to plants containing mutant mitochondria but generally not macroscopically variegated.
[3] Similar although distinct forms have been grouped under each type.
[4] Estimated from gross survey of all variegated individuals.
[5] Determinations of multichondric seedlings and mutant mitochondrial types based on only 61 plants.
[6] Index based on 106 seedlings.
[7] Dichondric rest homochondric.

plastids may become nearly normal in structure and color, this is purely a temporary phenomenon. No evidence could be obtained that the variable yellow-green type "back mutated" to normal. The extraordinary variability in color and vacuolation occurs in a characteristic manner and is as typical for this mutant as, *e. g.*, are large grana for the macrograna-type mutants.

Seedlings homochondric for the "variable yellow-green" type are capable of independent growth, although they develop very slowly at first. In homochondric-mutant branches the delayed development of chlorophyll, which is characteristic of the maturing leaves, is frequently more noticeable in the interveinal portions of the lamina. This gives the appearance of a veinal chlorosis and leaves the impression that an invasive virus or transforming factor is present. This is not the case, however, since in heterochondric plants transitions between normal and mutant areas were as sharp as in other plastid variegations (fig. 41). Furthermore, although a number of grafts were made between homochondric-mutant branches and normal clones, no transmission of chlorosis whatever occurred.

A number of other light green variegations, some resembling "light-green type 1" and some the "variable yellow-green type 1," differed from those just described only in minor details.

(*b*) Plastids with marked morphological abnormalities although some chlorophyll is developed: Examples are "macrograna type 1," "macrograna type 2," and "light-green type 2." The first two are remarkable because of the characteristic alterations in the size and number of grana [figs. 3, 4; for a detailed description see Woods and duBuy (*31*)]. The grana are greatly increased in size but reduced in number. At first they are nonvacuolate and contain few but very large grana. As the cell ages the grana break down and the plastid becomes characteristically vacuolate. This is an invariable sequence in development and has been repeatedly observed. Under the same conditions the normal plastids do not undergo changes in basic morphology (fig. 1). Tissue homochondric for "macrograna type 1" has a peculiar olive-green color, whereas "macrograna type 2" plastids impart a much lighter, more yellow-green color to the tissues. The "light-green type 2" mutant mitochondria form plastids which are pale green in young leaves, but fade to light yellow as the tissue ages. The plastids at first appear to be without grana, the pigment being diffuse throughout (figs. 5, 32). As the cell ages, these plastids become reduced in size and slightly vacuolate.

A pattern of plastid modification similar to that occurring in the macrograna mutants has been observed in *Nepeta* leaves infected with an unidentified virus (figs. 42–47). The condition here closely parallels that previously described in mosaic-infected tobacco (*31*). Such a pattern has never been observed in chloroses of nonvirus or nonchondriogenic nature.

(*c*) Plastids markedly reduced in size, and chlorophyll either absent or present in small amounts: Types of this subgroup develop very little or no chlorophyll although the carotinoids are present in noticeable amounts,

at least early in ontogeny. Examples are "cream type 1" (figs. 6, 50, 51) and "green-spotted cream" (fig. 36). For comparison of the various types described with normal leaves and plastids, figures 48 and 49, representing normal tissue, have been added.

2. *Mitochondrial types that usually affect growth.* Several mitochondrial types have been obtained that form very abnormal plastids or fail completely to differentiate into plastids. In one type, growth was stimulated and the plastids formed were supernormal in size and color.

(*a*) Mitochondria develop into plastids supernormal in size and color; cell division markedly stimulated in young actively growing leaves: The single example of this type, "supernormal type 1," appeared in a seedling derived from a cross between a polychondric plant homozygous for mutation-allowing *m* (female parent) and the homozygous stable clone (*MM*). The exceptional seedling 16–18 possessed some leaves with greatly enlarged, darker-than-normal green areas and young leaves with dark green blisters (figs. 65 and 66). A few shoots appeared to be normal. Other types of variegation were not present. Other seedlings in the progeny contained an assortment of the usual types, including those present in the female parent. At first the enlarged areas were suspected of being tetraploid, but measurements of cell size indicated that this was not the case because no significant increase in size could be detected. Cytological studies showed that leaf enlargement was clearly the result of increased cell divisions during leaf ontogeny rather than to an increase in cell size. On the other hand measurements of plastid size showed that the plastids in the dark green areas were larger than normal (table 4). While the rate of cell division in areas containing supernormal

TABLE 4.—*Comparisons of normal and supernormal areas in leaves containing supernormal type 1 chondriogenotype*

Structures measured	Number	Average	Minimum measure	Maximum measure	Supernormal/normal (average)
Width, base of marginal serrations:					
Normal	10	3.97 *mm*	3.00	5.00	1.52
Supernormal	10	6.07 *mm*	5.00	7.00	
Number, palisade cells across base marginal serrations:					
Normal	[1] 6	250 cells	233	274	1.47
Supernormal	[1] 6	369 cells	325	433	
Stomatal length:					
Normal	10	26.4μ	24.4	28.2	1.08
Supernormal	10	28.5μ	26.0	33.7	
Longitude cross-section area first layer palisade parenchyma:					
Normal	39	1232 sq.μ	795	1592	1.04
Supernormal	36	1290 sq.μ	795	1760	
Maximum linear dimension of chloroplast:					
Normal	165	5.55μ	3.26	7.60	[2] 1.27
Supernormal	163	7.06μ	4.34	9.23	

[1] Sections.
[2] Assuming a sphere, ratio based on cross-section area=1.60.

plastids was increased in leaves formed on young vigorous shoots, leaves with abnormal plastids, formed on older shoots, were darker-than-normal green and were sometimes not markedly changed in growth rate. Some leaves on these slow-growing shoots displayed an irregular mosaic pattern of normal and darker-than-normal green areas (fig. 67). Cells were found in these mosaic areas that contained chiefly normal-sized plastids (fig. 68), larger-than-normal plastids (fig. 69), or mixtures of the two (fig. 70).

Studies of meiotic-chromosome number in supernormal branches gave no evidence of polyploidy. The supernormal type 1 mutant presumably involved a chondriogene mutation leading to supernormal plastid development and stimulation of cell division. Unfortunately no data were obtained regarding the inheritance of the supernormal type plastids before the clone was lost.

(*b*) Plastids much reduced in size and without pigment: Two examples of this group are the white vacuolate type 1 (figs. 7, 52, 53) and the white vacuolate type 2 (figs. 8, 54–56). While both type 1 and type 2 white vacuolate plastids are devoid of chlorophyll, they can elaborate starch when living cells are maintained in the Aston flow cell and supplied for about 12 hours with nutrient solution plus 5 percent sucrose. Starch was demonstrated by staining with IKI solution.

The white vacuolate type 1 mitochondria differentiate into plastids of reduced size which become strongly vacuolate as the cell matures. They tend to replace rapidly the normal mitochondria in shoot ontogeny (fig. 11) and often retard leaf growth (fig. 52), but generally do not cause marked retardation in pigment formation and size of the nonmutant plastids of heterochondric cells (figs. 14, 23).

White vacuolate type 2 mitochondria may either fail to differentiate into plastids or in some cells form very small vacuolate plastids (fig. 8). Not only do the mitochondria of this type tend to displace the normal chondriome in successive cell divisions, but in heterochondric cells they exert a strongly inhibitory effect on the formation of pigment and on the growth of the nonmutant plastids in the same cell (figs. 71–73). The figures illustrate both the over-all retardation in cell development and the inhibitory effect on the nonmutant plastids.

(*c*) Mitochondria that do not develop into plastids; cell growth and differentiation markedly altered: The single example of this type appeared in clone 28–1 (fig. 57), which was derived from a cross between the original mutable plant and the stable homozygous clone as pollen parent (see second part of this paper on the mutation-allowing gene). Since this type ("mitochondrial white") was apparently totally unable to differentiate as plastids (fig. 58), its history is described in some detail. The data are particularly significant in connection with the concept of the unity of the chondriome in plants [see Newcomer (18)] and in relation to the extremes to which mitochondrial mutation can go.

Cytological analyses were repeatedly made of leaves of all ages containing the "mitochondrial white" mutant mitochondria. Affected cells were greatly reduced in size and were more isodiametric than normal cells.

Plastids were never observed in any of these cells. The mitochondria were clearly differentiated by vital staining with Janus green B (fig. 58). This staining was dependent upon an adequate supply of oxygen and was reversible. This type is unique in that it represented a change of such character that all ability to form plastids was lost, although the mutant mitochondria could maintain the life of the cell provided photosynthate was supplied from the green portions of the plant.

The terminal shoot of seedling 28–1 first displayed the pure white, stunted tissues characteristic of this type. When first observed periclinal stabilization of the mutant mitochondria had already occurred. No other seedling of this progeny displayed the same type although an unusual number of growth-distorting derivatives was present. Of a total population of 78 plants, 64 contained one or more foci of mutant mitochondria, the usual types of mutants being in evidence. Three of the 64 plants carried growth-distorting factors in addition to ordinary types of variegation-inducing plastids. Clone 28–1 was propagated by cuttings. These and the original stock continued to produce tissue containing the "mitochondrial white" type, and in addition many nonvariegated stable green branches. After several months new vigorous shoots developed from the original crown, and several of these carried the "mitochondrial white" mutant mitochondria in periclinally stabilized form. Foci of the "mitochondrial white" mutant mitochondria were maintained for about 1 year.

3. *Mitochondrial derivatives not cytologically recognizable, but detectable by virtue of their profound influence on growth and differentiation of the cells containing them.* While most of the mitochondrial types obtained in *Nepeta* have been capable of existing in the homochondric state, and in fact tend to displace the normal chondriome in successive cell divisions (fig. 11), certain derivatives behave in a different manner. These agents (table 1) seem to be either indistinguishable from undifferentiated mitochondria or are subvisible. They have appeared only in progeny of crosses in which plants homozygous for mutation-allowing *m* were used as the female parents. Their pathogenic action is closely analogous to that of viruses of the growth-distorting mosaic types. However, repeated attempts at graft-transmission have been completely negative (table 2). That the methods of grafting were adequate for detection of viruses was proved by the successful transmission of an unidentified virus found naturally infecting wild plants of *Nepeta* (figs. 45–47). These tests were carried out in an isolated greenhouse to avoid chance contamination of the other experimental stocks. Further evidence for the absence of transmissible viruses in the *Nepeta* stocks used in the chondriogene studies was provided by employing dodder grafts (*Cuscuta* sp.) between variegated mutable clones (*mm*) and nonvariegated homozygous stable (*MM*) plants. Connections were established between mutable *Nepeta* plants and healthy Turkish tobacco plants. All of the tests with dodder failed to give any evidence of transmission of variegation-inducing or growth-distorting factors from the mutable *Nepeta* plants.

Plants affected with these noninfectious growth-distorting factors, when first observed, usually had some shoots bearing distorted leaves with very irregular white mosaic areas (figs. 59, 60). In some regions the entire mesophyll of the leaf was reduced to a single layer of very abnormal cells (fig. 62). Plastids were entirely lacking in some cells, all of the chondriosomal elements being present in the mitochondrial form. These stained vitally with Janus green B. In other cells the number of plastids was reduced to only a fraction of the normal complement (figs. 62–64). Some leaves contained areas with subacute symptoms only (fig. 61). Maturation of plastids in these areas was apparently nearly normal in some cells and markedly abnormal in others. The general picture in cells containing the agent was that of a variable suppression in the development of the normal chondriome. All gradations in plastid suppression were observed.

The chronic-symptom pattern in some of these growth-distorting types was strikingly like that characterizing virus-induced rugose mosaics in other plant species. This is well illustrated by clone 35–23 (figs. 74, 75). The effect of these mutations on fertility are described below.

Another growth-distorting factor of an unusual type is the "spider" mutant (figs. 76–79). While cytological studies are incomplete, this type probably belongs in the same group of mutants as 35–23. The mutant does not reduce the chlorophyll content but its presence is evidenced by a slight narrowing of the leaf associated with a rolled and glossy appearance (fig. 78). The corolla is shortened so that the styles appear long and give a "spidery" appearance to the inflorescence (figs. 76, 77). In some plants the "spider" factor occurred throughout the whole soma, whereas in other instances only one or two branches were involved. In the seedling 140–9, one main branch carried the spider factor (figs. 76–78), whereas other branches of the same plant, though lacking this factor, carried other types of mutants (figs. 80–82). The behavior of the spider mutant in sexual inheritance is described below.

In the course of this investigation a number of other mutations, more or less resembling some of the types described above, have been encountered (figs. 33–35). It seems probable that the total number of possible chondriogene mutations in *Nepeta* far exceeds the number recorded here. The situation may well be comparable to that occurring in tobacco-mosaic virus regarding which Bawden [(*2*) p. 107] has stated, ". . . there is good reason to consider that new strains are constantly arising, the number that could be identified is probably limited more by experimental techniques and investigators' perseverance than by the total number possible."

II. Genetical

A. *Independence of the occurrence of different mitochondrial types from the nuclear genetic constitution of the plant.* In order to understand the implications of the results reported in the second part of this paper, the question should be raised: How does one know that the mitochondria are the elements that mutate?

Before Dr. Walker supplied the variegated *Nepeta* which provided the basis of the present study, variegations were found accidentally in nature. For instance, Mr. F. B. Winkler, cultivating Antirrhinums, occasionally supplied plants with variegated leaves belonging to either one of two types. These variegations, occurring at the rate of 1 per several thousand, were inherited matroclinously (*29*). Another variegated plant which occurred among a stand of thousands of Xanthiums, demonstrated a similar behavior. Single individuals of over 40 plant species were furnished, in which variegational areas occurred on only 1 or 2 leaves of each individual. Many of these disappeared without the development of variegation in the sexual organs.

In the course of our study, as stated before, also a great number of natural stands of *Nepeta* was studied for the possible occurrence of variegation, in order to establish that mitochondrial mutation was not a frequent occurrence. For the same reason, 1 of the normal plants of such a stand was removed to the greenhouse and propagated both sexually and asexually. This clone, which was also used in hybridization studies, will be referred to as "stable" green. The 239 seedlings obtained by selfing this clone or its sexually produced progeny were all free of chondriogene mutation (table 3, cross-type 1). However, after about 3 years of extensive vegetative propagation 1 instance of chondriogene mutation was observed in the original clone. This involved a single locus of approximately 15 square millimeters in 1 leaf. Cytological examination of this area revealed distinctly abnormal light-green mutant plastids occurring exclusively in some cells (homochondric mutant), and mixed in varying proportions with normal plastids in other cells (heterochondric). The bud in the axil of this single variegated leaf was induced to elongate into a branch of several nodes, but no further variegated areas appeared. Apparently the mitochondrial mutation had occurred in cells that were not a part of the continuing meristem of the plant. This single observed case of mitochondrial mutation, which occurred about 3 years after the beginning of greenhouse culture of the clone, indicates that in keeping with all other thoroughly investigated species, "spontaneous" mutation of chondriogenes can occur in the absence of special mutation-allowing or "mutagenic" factors in the nucleus. The frequency of such mutation, however, is so low that for the purposes of the present investigation it can be considered practically zero.

Whereas results obtained with the "stable green" clone allowed the conclusion that normally mitochondrial mutation was not a frequent occurrence, the behavior of the mutable clone of *Nepeta*, derived from 1 variegated plant, demonstrated that under certain conditions a high rate of chondriogene mutation can occur. Repeated attempts to establish nonvariegated clones by asexual propagation of apparently normal green shoots of this original variegated clone invariably failed. Even though cuttings were rooted from branches in which no trace of variegation was visible, some of the shoots that subsequently developed always became variegated. Cytological examination of such variegated areas revealed

typical heterochondric and homochondric cells. When apparently normal shoots of this original mutable clone were self-pollinated, all of the seedlings invariably became variegated before maturity. Thus, in 1 experiment 40 such seedlings were at first predominantly normal green, but by the time the plants were approximately 20 cm. tall all had become variegated in one or more areas. Furthermore, the markedly different types of variegation (light green, cream, white, *etc.*) described above, appeared in the seedlings, several types sometimes occurring in the same leaf. An apparently normal shoot of 1 of these variegated seedlings was self-pollinated, and from it were derived 50 seedlings all of which became variegated in the same manner as the progeny from the original clone. Other crosses gave similar results (*cf.* table 3, cross-type 8). When variegated shoots of the original mutagenic clone or of its progeny were self-pollinated, all of the seedlings were also variegated (table 3, cross-type 12). Similar results were obtained when only the female was visibly variegated (table 3, cross 13). These results suggested that the original plant might be homozygous for a mutation-allowing gene or genes, in some way associated with multiple mutations in the chondriogenes.

In order to establish that mutations occur at the mitochondrial level, but that these mutations occur frequently only in the presence of a mutation-allowing nuclear genom, and only then are producible at will, a number of criteria must be met. Such a mutation should 1) perpetuate itself in the plant of its origin, once having occurred under the conditions that obtain in the presence of a certain nuclear genom, 2) will perpetuate itself when introduced in a linea of cytoplasm in which variegation did not occur previously or occurred rarely, 3) will perpetuate itself in such a linea when propagated vegetatively, 4) will segregate according to chance distribution in dividing cells, 5) will remain cytologically identifiable, 6) will be sexually inherited in a non-Mendelian manner, and 7) can be demonstrated, with the techniques available, not to be of virus nature. These criteria have been met in *Nepeta* and are reported below, although not in the same order.

B. *Inheritance of the mutation-allowing character.* Reciprocal crosses between flowers of *nonvariegated* shoots of mutable clones and those of the stable-green clone gave 3 types of seedlings. One type remained nonvariegated during observation periods of 1 to 2 years. These were the only type produced when the *nonvariegated* shoots of the mutable clones served as the male parent (table 3, cross 3). A second type, which occurred regardless of the direction of the cross, was variegated, and usually developed one or more green shoots that did not become variegated even after several years of vegetative propagation. The third type, which occurred only when the female was variegated and regardless of whether the male was variegated or not, appeared to be entirely lacking in normal plastids (plastids were light green, white, *etc.*). These, with rare exceptions died after several days. The results of these crosses suggested that the tendency to mutation was entirely recessive to a character, or characters, carried by the normal green clone, since all green shoots produced on either

totally green or on variegated plants showed no evidence of recurrent mitochondrial mutation. The occurrence of variegated progeny when macroscopically variegation-free shoots of the mutable clones were used as females (table 3, cross 7) can be explained on the basis of trace contaminations of the mutable soma with mutant mitochondria. As is pointed out below, mutation of mitochondria apparently can occur in the gametes, but at a very low frequency.

Preparatory to further hybridization tests, meiotic analyses were made of the anthers of the original stable green and mutable variegated clones. Analyses were also made of some of the F_1 plants of the reciprocal crosses between these clones. In all cases meiotic behavior corresponded to that of a normal diploid with 32 chromosomes (2n). At first metaphase, 16 bivalents, and at second metaphase, 16–16 distributions of univalents could be found. Tetrad formation was regular, and there was no evidence of pollen abnormalities.

Table 5 summarizes the results obtained after self-pollination of 10 stable nonvariegated plants, derived from the cross *mutable* × *stable*, or its reciprocal. Self-pollination of stable nonvariegated shoots of 14 otherwise variegated plants gave similar results (table 6). They show that the tendency toward a high rate of chondriogene mutation is inherited as a simple Mendelian recessive, good agreement with a 3:1 ratio obtaining in most instances. It is also apparent from the data that inheritance of the mutagenic gene through either the male or female gamete has no significant influence on the frequency of mutable seedlings in the progeny of the heterozygous plants so formed (table 5). Further confirmation

TABLE 5—*Inheritance of the recessive mutation-allowing gene, m, in 10 different progenies obtained by selfing homochondric normal plants derived from reciprocal crosses between stable and mutation-allowing clones (MM×mm and reciprocal). The parent from which each heterozygote inherited the mutation-allowing gene is indicated*

Cross	Seedlings			Expected 3:1 ratio		Mutation-allowing gene introduced through indicated gamete
	Total	Normal	With mutant mitochondria	Normal	With mutant mitochondria	
1	125	101	24	94	31	Female.
2	107	77	30	80	27	Do.
3	109	81	28	82	27	Do.
4	63	41	22	47	16	Do.
5	125	97	28	94	31	Male.
6	85	61	24	64	21	Do.
7	47	35	12	35	12	Do.
8	144	96	48	108	36	Do.
9	71	47	24	53	18	Do.
10	201	144	57	151	50	Do.
Subtotal	404	300	104	303	101	Female.
Subtotal	673	480	193	505	168	Male.
Total	1,077	780	297	808	269	Female, male.

was provided by reciprocal crosses between plants heterozygous for the mutation-allowing gene and the original stable homozygous clone. All

progeny of these crosses behaved as stable types. On selfing normal shoots of such progenies these were found to be either homozygous dominant or heterozygous. The latter, when selfed again, gave 3:1 ratios for stable: mutable. The recessive mutation-allowing gene has been designated by *m* and the dominant stabilizing factor by *M*. These symbols have been used in all tables.

TABLE 6.—*Inheritance of the recessive mutation-allowing gene, m, in 14 different progenies obtained by selfing homochondric normal shoots on plants heterozygous for the mutation-allowing gene (Mm). These heterozygous plants all possessed some heterochondric tissue in branches other than those from which seed was obtained (see last column)*

Cross	Seedlings			Expected 3:1 ratio		Source[1] mutation-allowing gene	Types of mutant mitochondria in variegated branches, mother plant
	Total	Normal	With mutant mitochondria	Normal	With mutant mitochondria		
1	150	116	34	112	38	Male, self	White vacuolate 1.
2	58	43	15	43	15	Female	Mitochondrial white.
3	97	71	26	73	24	Female, female.	Cream.
4	63	48	15	47	16	do	Do.
5	41	31	10	31	10	do	Do.
6	70	56	14	52	18	do	Variable yellow-green.
7	62	47	15	46	16	Male, female	Do.
8	161	123	38	121	40	Female, female.	Stunting mosaic.
9	84	68	16	63	21	do	Light green, 35–23 growth-distorting factor.
10	39	30	9	29	10	do	Cream, "spider" growth-distorting factor.
11	20	15	5	15	5	do	Medium green.
12	16	12	4	12	4	Female, female, self.	Light green, 34–2 growth-distorting factor.
13	20	16	4	15	5	Female, female.	Light green.
14	69	49	20	52	17	do	Cream.
Total	950	725	225	711	239		

[1] The mutation-allowing gene *m* was introduced into the *Mm* parents *via* one or more crosses from *mm* plants as indicated.

C. *Chondriogene mutation in gametes.* Selfed nonvariegated plants, heterozygotic for *m*, regularly gave 3:1 ratios for *stable: mutable* plants, in which the *mm* offspring was variegated, and the homozygotic *MM* and the heterozygotic *Mm* offspring were stable green. However, among the variegated seedlings which were all expected to be homozygotic *mm*, occasionally one was found that produced, on further cultivation, "stable green" shoots, thus behaving as if its genetic constitution contained *Mm*. For example, such a seedling, carrying a white vacuolate type of mutant plastids, was found among the offspring of 9 plants which were carefully checked for the absence of any variegation. These 9 plants had been

proved previously to be heterozygotic for *m*. The parent of this particular seedling had previously, on selfing, given 57 offspring that became variegated and 144 that remained normal (expected ratio, 50: 151), indicating that the parent indeed was heterozygous for *m*. The total progeny of the 9 parent plants was 905, of which 658 seedlings were stable green and did not develop any variegation, and 247 showed variegation (expected ratio, 679:226).

The one exceptional variegated offspring of the nonvariegated parent proved previously to be heterozygotic for *m*, developed a number of stable green shoots, indicating that its genetic constitution was *Mm* and thus should have been nonvariegated. The question thus arose concerning the origin of a mutant plastid in a heterozygous seedling from a heterozygous *green* parent. A number of possibilities presented themselves. An *m* gene in the *mm* combination might have back-mutated to *M*. However, homozygous mutable clones (*mm*) have been asexually propagated for several years without the development of stable shoots. This indicates that if such back-mutation occurs at all it must be very rare.

Another possibility would be that a spontaneous mitochondrial mutation occurred. However, such mitochondrial mutations occur at an extremely low rate in the presence of dominant *M*, whereas variegated heterozygous seedlings were obtained several times among the offspring of self-pollinated, nonvariegated, heterozygotic parents. Various types of mutations were found under these conditions.

It is unlikely that this exceptional plant arose through somatic elimination of the *M* locus in an *Mm* plant because this would have resulted in the formation of somatic tissue with unstable chondriogenes. This seedling was of the stable type.

These observations, coupled with the fact that the variegational types of these exceptional seedlings were stable throughout their vegetative structure, lead to the third, most probable answer that mutation of the mitochondria occurred in the absence of *M* during the haploid stage of the gametes carrying *m*.

D. *Chondriogene stability in heterozygous clones.* While the results of the various hybridization experiments indicated that the mutation-allowing gene *m* was recessive to *M*, a number of clones were selected for rigorous testing by long-continued vegetative propagation. The following history of clone 16–7 is typical of a number of such tests. The clone was derived from a cross in which mutation-allowing *m* was introduced through the female gamete. Seedling 16–7 was free of variegation from its first appearance. All leaves formed were examined with a 4 × magnifying lens, and at no time were any traces of variegation observed. About 4 months later a number of flowers were self-pollinated, and of the 125 seedlings obtained 24 eventually became variegated. This proved the clone to be heterozygous for *m*. Twenty days later the original plant, which was still variegation-free, was cut back to force new growth. A vigorous development of new shoots ensued, and 19 days later these

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were carefully examined for foci of mutant plastids. A total of 165 mature leaves and the young leaves of 57 terminal or axillary shoots were examined and found to be free of variegation. The clone was then expanded by vegetative propagation and maintained for about 1 year. No traces of variegation appeared. This type of careful checking was carried out with similar results on a number of other variegation-free clones proved by genetic analysis to be heterozygous for *m*. The results establish the complete dominance of *M* over *m*.

E. *Inheritance of mutant mitochondria.* Reciprocal crosses were made between nonvariegated and variegated plants of *Nepeta* to determine in more detail the manner in which the mutant (variegation-inducing) mitochondria are inherited (table 3). The results showed that the inheritance was non-Mendelian and, as would be expected, was chiefly through the female gametes. No characteristic ratios appeared. However, when the female parent was strongly variegated (50 to 100 percent of the mitochondria mutant) and the male normal, the progeny were very strongly variegated, often with mutant plastids only. The reciprocal crosses yielded progeny that were at first entirely free of mutant mitochondria or nearly so. Table 3, column 9, gives an approximate measure of the quantitative transmission of mutant mitochondria. The measurements were made chiefly in well developed plants (circa 10 to 20 cm. tall) and are expressed as an index number obtained by multiplying the percentage of the total population that contained mutant mitochondria by the percentage of such plants that had an estimated 20 percent or more of the soma variegated. The results demonstrated that many more mitochondria enter the zygote from the cytoplasm of the egg cell than from the male gametes. It was clear, however, that male cytoplasm did in some instances take part in the formation of the offspring [see Anderson, (*1*)]. When seedlings receiving such male cytoplasm were examined in the early stages of growth (circa 1 to 5 cm. tall) it often appeared as though transmission of mutant mitochondria had not occurred. However, when the seedlings were grown for several months the foci of mutant plastids became apparent.

F. *Stability of mutant mitochondrial types.* It was of considerable interest to determine whether a given mutant mitochondrial type would retain its unique characteristics (*a*) in long-continued asexual reproduction, and (*b*) in sexual reproduction. A number of experiments were performed to test these points.

Clones carrying characteristic mutant chondriogenotypes were propagated by cuttings for periods ranging from 6 months to over 1 year. Table 7 lists some of the mutant mitochondrial types, or derivatives, maintained in this way. In every case the abnormal types were permanently "fixed," *i. e.*, capable of continuous reproduction. Of course any mitochondrial type may be lost through segregation, but it was nearly always possible to maintain them in some part of the plant. Such "cultures" of mutant mitochondria are in most instances dependent upon the presence of sufficient numbers of normal mitochondria to form photo-

synthetically active normal chloroplasts (figs. 11 and 12). The results of these experiments showed that mutant mitochondrial types of widely differing properties maintained their unique characteristics for the life of the clone.

TABLE 7.—*Persistence of mutant mitochondrial types, or their derivatives, in asexual reproduction of the soma*

Mutant mitochondrial type or derivative	Genotype [1]	Period of vegetative propagation [2] (months)
Supernormal type 1	*Mm*	6
Light green type 1	*Mm*	9
Light green type 2	*Mm*	6
Light green type 3	*Mm, mm*	15
Macrograna type 1	*Mm, mm*	12
Macrograna type 2	*mm*	12
Variable yellow-green type 1	*MM* or *Mm*	24
Green-spotted cream	*Mm*	33
Cream	*MM, Mm*	14
White vacuolate type 1	*Mm, mm*	12
White vacuolate type 2	*mm*	12
Mitochondrial white	*Mm*	10
34–2 growth-distorting factor	*Mm*	40
35–23 growth-distorting factor	*Mm*	41
"Spider" growth-distorting factor	*Mm*	12

[1] *M* represents dominant gene which completely eliminates mutation-allowing influence of recessive *m*. Mutant mitochondria or derivative carried in 1 or more clones as indicated.
[2] Periods are minimal. In all cases abnormal mitochondrial type or derivative was perpetuated for the life of the soma.

Flowers were allowed to develop on branches bearing various chondriogenotypes and crosses made in such a way that the offspring would usually contain the stabilizing gene *M*. In some instances, however, the progeny were homozygous for mutation-allowing *m*. The data (table 8) show that mutant mitochondrial types can be passed through the gametes without loss of characteristic abnormalities. While new mutations may have arisen in haploid gametes carrying mutation-allowing *m*, or in seedlings homozygous for *m*, the frequency of occurrence of gametic mutation, on the one hand, and new mutation in *mm* seedlings on the other, was too low for the types concerned (see table 3, crosses 7 and 8, white types) to account for the results obtained on any basis other than gametic transmission of preexisting mutant mitochondrial types. For example, in the last cross of table 8 about 90 percent of the offspring carried mutant mitochondrial types present in the female parent. The appearance of these types through new mutation, on the other hand, was rare, as can be seen from table 3 (all white types together having a frequency of only 3.8 to 11.4). Many other crosses repeatedly demonstrated the same fact.

G. *Independence of survival of mutant mitochondrial types from the gene m.* While it was obvious that a high frequency of chondriogene mutation occurred only in plants homozygous for *m*, there was no evidence that survival of mutant mitochondrial types was in any way dependent upon this gene. Mutant mitochondria propagated freely, and apparently

TABLE 8.—*Persistence of mutant mitochondrial types in sexual inheritance*

Genotypes and mutant chondriogenotypes of parents				Seedlings		
Female		Male		Number	Percent with parental mutant mitochondrial types	Probable genotypes
Nucleus	Mitochondria	Nucleus	Mitochondria			
mm	Light green 2	*MM*	Normal	56	23.2	*Mm*
mm	Light green 3	*MM*	do	40	97.5	*Mm*
mm	White vacuolate 2	*MM*	do	8	62.5	*Mm*
mm	White vacuolate 1	*MM*	do	13	White vacuolate 1 23.0	
	Macrograna 1				Macrograna 1 7.7	*Mm*
					Both types 15.4	
					Total 46.1	
mm	Cream	*mm*	Like female	19	89.4	*mm*
MM	Normal	*mm*	Cream	25	24.0	*Mm*
MM or *Mm*?	Variable yellow-green	*MM*	Normal	13	100.0	*MM* or *Mm*
MM or *Mm*?	do	*MM* or *Mm*?	Like female	32	100.0	*MM* or *Mm*
Mm	Green-spotted cream	*MM*	Normal	6	50.0	*MM*, *Mm*
mm	White vacuolate 1	*mm*	Like female	18	White vacuolate 1 22.2	*mm*
	White vacuolate 2				White vacuolate 2 16.6	
	Macrograna 1				Macrograna 1 5.5	
	Macrograna 2				Macrograna 1, 2 5.5	
					White vacuolate 1, 2 / Macrograna 2 5.5	
					Macrograna 2 / White vacuolate 2 16.6	
					White vacuolate 1 / Macrograna 1 11.1	
					White vacuolate 2 / Macrograna 1, 2 5.5	
					Total 88.5	

indefinitely, in heterozygous clones where *M* was dominant. To determine whether the presence of mutation-allowing gene *m* was necessary for the survival of mutant mitochondrial types, crosses were made between strongly variegated females (90 to 100 percent variegated) heterozygous for *M* (*Mm*), with pollen from the nonvariegated homozygous stable clone (*MM*). Fifty percent of the progeny of such crosses should have been heterozygous (*Mm*) and 50 percent homozygous dominant (*MM*). The results showed that of 126 seedlings 109, or 86 percent, were variegated (table 3, cross 10) and 17, or 14 percent, were green, indicating that a number of variegated plants occurred in the progeny that were homozygous dominant (*MM*).

To test directly for the occurrence of mutant mitochondria in homozygous dominants (*MM*), less strongly variegated females were crossed with the stable clone so that enough normal mitochondria would be present to allow both survival of the seedlings and the development of nonvariegated shoots for genetic testing. Some of the variegated progeny obtained in this way were grown until one or more nonvariegated shoots appeared on each plant. Flowers on these green shoots were selfed, and the seedlings obtained were grown until well beyond the stage required to give reliable determinations of chondriogene mutability. For example, selfing of flowers of normal green branches of 1 plant, which on other branches carried foci of cream variegation, produced 135 seedlings. All seedlings remained green indefinitely. In the parent plant and the clone derived from it, the cream foci persisted (clone maintained for over 10 months).

Another plant with extensive foci of variable yellow-green mutant plastids produced normal green shoots with flowers, which on selfing yielded 140 seedlings, all of which remained free of variegation. These results demonstrate that the parent plant did not carry the mutation-allowing gene *m*, but nevertheless retained the variegation-inducing foci in it and its clone for life. It can be concluded that the presence of the mutation-allowing gene *m* is not necessary for the survival and reproduction of mutant mitochondrial types.

H. *Influence of mutant mitochondrial types upon fertility.* The growth-distorting "mitochondrial white" type 28–1 (see p. 1114) was first observed in chimeric, more or less periclinal form. This periclinal stabilization involved the subepidermal or first mesophyll layer of tissue which was homochondric for the mutant mitochondria. Thus, following flower development it was expected that most of the gametes would be homochondric mutant. Flowers developed on such periclinal shoots were generally both ovule and pollen sterile. These results show that fertility can be affected by the presence of abnormal mitochondria. That this effect on fertility is indeed dependent on the presence of this particular mutation is further demonstrated by the following observations.

As a result of periclinal division of the dermatogen, which was homochondric normal, dark green sectors of homochondric-normal mesophyll were sometimes formed [see Dermen (*5*)]. Flowers from stem tissue of such constitution were fertile. Genetic analysis of variegation-free green

branches proved the clone to be heterozygous for the mutation-allowing gene *m*. Thus of 58 seedlings, obtained by selfing the flowers produced on such a shoot (table 6, cross 2), 15 developed typical mutant mitochondria of several types and 43 remained free of variegation. *None* of the mutant mitochondrial types bore any resemblance to the "mitochondrial white" type.

While all of the growth-distorting agents were at first associated with complete ovule and pollen sterility, long-continued propagation by cuttings resulted, in one case (clone 35–23), in a return to limited fertility. Since the results may have considerable significance with respect to the problem of virus origin, they are presented in some detail.

The stunting factor in seedling 35–23 was originally localized in only part of the soma, and cuttings were rooted from both normal and abnormal branches (figs. 74, 75). It should be noted that the symptom pattern in affected shoots was so irregular and diffuse as to suggest that limited intercellular invasion might have occurred during ontogeny, although proof of such invasiveness is lacking. The symptoms occurring in shoots affected with this agent were symptomatically indistinguishable from those occurring in other plants affected with typical rugose-mosaic-type viruses. The abnormal shoots at first grew very slowly, and were both pollen and ovule sterile. During the next 2 years, however, a slow increase in vigor occurred. After 2 years of vegetative propagation the affected cuttings grew better than the normal ones. Although growth distortion and a mosaic-like chlorosis continued to characterize the clone, limited fertility was found. Table 9 summarizes the results of a genetic analysis of both

TABLE 9.—*Hybridizations involving plants containing either the 35–23 growth-distorting factor or the "spider" growth-distorting factor.*

Cross [1]	Plants with mutant mitochondria	Plants with stunting factor	Total	Mutant mitochondrial types appearing in seedlings
Mm (stunt) × self	12	0	47	Mostly cream.
Mm (stunt) × *MM* (normal)	1	0	42	Small focus of a white or very light cream type.
MM (normal) × *Mm* (stunt)	2	0	95	1 cream, 1 light green.
Mm (spider) × self	18	0	79	Light green, cream, white vacuolate, macrograna, *etc.*
MM (normal) × *Mm* (spider).	0	0	13	None.

[1] Genotypes with respect to *M* and *m*, chondriogenotypes indicated in parentheses (35-23 referred to as "stunt").

normal and stunt-affected clones. The results show, as would be expected from the parentage of seedling 35–23, that both normal and stunt-affected clones were heterozygous for the mutation-allowing gene (*Mm*). It is especially noteworthy that in no case was the 35–23 growth-distorting character transmitted sexually, or did it appear in any of the homozygous mutable seedlings. In general, the agent behaved as a non-seed-borne, rugose-mosaic-inducing cytoplasmic factor of noninfectious character.

The "spider" growth-distorting factor apparently caused some reduction in seed set but much less than in the case of clone 35–23. While the spider factor was propagated indefinitely in asexual reproduction (table 7), like the 35–23 growth-distorting factor it was not transmitted sexually (table 9).

I. *Association of mutations.* As the study progressed it became evident that most branches carrying macrograna type 1 mutant plastids also contained foci of white vacuolate type 1 plastids, the former being at first more extensively distributed (table 10). In mutable (*mm*) plants the frequent occurrence of small white flecks in the olive-green areas containing macrograna type 1 mutant plastids suggested that the white-vacuolate type 1 chondriogenotype was arising secondarily from the macrograna type 1. In this connection the situation in mutable (*mm*) clone 45–77 (table 10) is of interest. This clone developed a number of

TABLE 10.—*Association of macrograna and white vacuolate types of mutant plastids in plants homozygous for the mutation-allowing gene m*

Mutable plant	Relative amount macrograna types	Relative amount white vacuolate types	Parent of mutable plant	
			Genotype [1]	Chondriogenotype
12–58–197	4+ (type 1)	1+ (type 1)	*Mm*	Normal.
12–58–179	3+ (type 1)	1+ (type 1)	*Mm*	Do.
78–2	3+ (type 1)	2+ (type 1)	*Mm*	Do.
45–77	3+ (types 1 and 2)	2+ (types 1 and 2)	*Mm*	Do.
23–3A [2]	3+ (type 1)	2+ (type 1)	*mm*	Cream, normal.
23–3B [2]	3+ (type 1)	1+ (type 1)	*mm*	Do.

[1] With respect to mutation-allowing gene *m*.
[2] Separate shoots A and B on 1 plant.

mutant types and among these were foci of a new form: macrograna type 2 (fig. 4). In leaves containing macrograna type 2 the associated white vacuolate mutant was also of a new type, white vacuolate type 2 (fig. 8). In spite of obvious similarities between the two macrograna types and the two white vacuolate types, respectively, each is distinct. The results indicate that specific chondriogene mutations occur that control certain characteristics of the plastids. These mutations seem to occur in a certain sequence in which the first mutation conditions the expression of the subsequent mutation.

DISCUSSION

The autonomous hereditary (extranuclear) nature of plastids has been accepted by many investigators (*13*, *14*, *16*, *20*, *32*). However, the possibility has been suggested (*22*) that cases of so-called plastid mutation may actually be related to a permanently changed cytoplasmic condition in which normal plastid growth is impossible. This latter view leads to the further suggestion [(*22*), p. 203] that some type of extramitochondrial (or plastid) cytoplasmic components, "plasmagenes," may be involved. The present results strongly support the first view but not the second. They demonstrate that many distinct types of mutant mitochondria can

be recognized by their plastid forms in the cells, and all possible combinations observed without transition forms between normal and mutant types. Such transition forms might be expected if diffusible extramitochondrial cytoplasmic factors were responsible for the plastid abnormalities. Many mutant mitochondrial types are perpetuated in sexual inheritance although some derivatives (*e. g.*, 35–23 growth-distorting factor) are not. All mutant types and derivatives are perpetuated in asexual inheritance. Some of these abnormal mitochondrial derivatives cause pronounced derangements in cell growth and function. *They constitute the continuing cause of distinct neoplasia.* Koch's postulate has been fulfilled to the extent that, by sexual transfer in non-Mendelian fashion, abnormal mitochondria have been introduced into a cell with a certain nuclear genom, and the disease symptoms studied. Thereupon, the disease-causing agents have been removed by allowing them to segregate out in clonal multiplication, with the result that the disease symptoms disappeared. Reintroduction by sexual crossing caused reappearance of the symptoms.

It has already been shown that mutant mitochondria and plastids display characteristic enzymic abnormalities (*8, 30*). The present results show that nuclear factors may greatly accelerate the mutation rate of the chondriogenes, but that such factors are not necessary at all for the survival of the mutant mitochondria or their derivatives, once they have appeared. Rhoades (*21*) has reported a similar situation in *Zea* with the important difference that multiple types of chondriogene mutations apparently were not obtained. This circumstance may have led him to suggest that the plastid abnormality might be under direct control of a self-reproducing entity of nuclear-gene origin or under indirect control of an entity in the cytoplasm produced under influence of the nuclear genom and affecting in its turn the plastids. These alternatives are based on the occurrence of pale green plastids in cells adjacent to white cells. Rhoades [(*22*) p. 205] suggests "that there is some substance in the colorless cells, *responsible for the abnormal plastids found there*, which is able to diffuse across cell membranes and impair chlorophyll development in normal cells." [Italics ours.] A more probable explanation seems to be that the metabolism of *mutant* plastids liberates toxic products which act *secondarily* on the plastids of normal types. We have observed a similar phenomenon in certain "white" mutants in *Nepeta* (figs. 71–73). It is known that disturbances in oxidizing enzymes (*23, 24, 26*) occur in variegated tissues. The products of the oxidation of phenolic compounds, such as occur in plant tissues, can result in destruction of chlorophyll (*9, 31*). According to this view the primary cause of albinism is the mutant chondriogene, whereas the substance affecting nonmutant plastids is a secondary, diffusible product, *e. g.*, a quinone-like product. In view of these observations, there is no need to postulate a diffusible "plasmagene" in order to explain the conditions observed in *Zea* by Rhoades.

In *Nepeta* the very wide range of chondriogenotypes obtained suggests that the nuclear gene *m* acts more as a mutation-stimulating (or per-

mitting) agent rather than as a direct cause. Because the mechanism of action is not known, the less committing term "mutation-allowing" has been used in place of "mutagenic," in order to denote its influence on the complex of hereditary chondriogenes which are carried by the mitochondria. In this connection it is of interest to note that Bawden [(*2*) p. 116] has proposed a similar complex of hereditary factors carried by viruses.

The complete absence of graft-transmissibility, either of the mutation-allowing tendency or of any of the abnormalities, demonstrates that transmissible viruses cannot have been responsible for the effects noted. Viruses can, however, cause nearly identical plastid modifications (*29*, *31*).

In the present study it has been possible to obtain, in one species, abnormal mitochondrial derivatives that range from only slightly abnormal mitochondria (as judged by the plastids formed from them) to forms developing into highly abnormal plastids, to forms unable to develop into plastids at all, and finally to agents that act on the cell in a manner closely similar to viruses. These agents are apparently no longer able to function as a chondriome.

The accumulated evidence indicates that mitochondria have a complex chondriogenic constitution. For example, there appear to be specific factors affecting development and retention of plastid pigmentation, size of the grana, plastid vacuolation, influence of the abnormal mitochondria on cell growth and function, *etc.* Differences in specific enzyme activities have been reported previously (*8*, *30*).

Many types of plastids can occur in one cell, whereas related viruses are mutually exclusive under certain conditions. This seems contradictory to the view that viruses and mitochondrial derivatives have a common origin. The following considerations, however, should be taken into account. It was shown previously that, following primary infection, the multiplication of tobacco-mosaic virus requires the functioning of cyanide-sensitive respiratory enzymes (*27*, *28*). Later it was shown (*8*) (du Buy *et al.*, 1950) that those enzyme systems are carried on the mitochondria. In this connection some statements by Bawden (*2*) are of interest: "(Virus) antagonism is probably restricted to competition for suitable substrates . . ." and ". . . as a preliminary to multiplication, virus particles must become attached to some cell constituents and . . . there are only limited numbers of such sites in each cell. . . ." On the basis of the above-mentioned studies, mitochondria and their derivatives may be these sites. Virus multiplication and the multiplication of strongly aberrant mitochondrial derivatives (*e. g.*, the *Nepeta* 35–23 type of agent) could thus be expected to compete for certain loci on normal mitochondrial elements, and be limited in their multiplication by the number of mitochondria present, whereas the less abnormal mitochondria can duplicate themselves independent of the presence of normal mitochondrial elements, subject to other limiting factors for their multiplication.

The failure to transmit such virus-like agents by grafting can mean that chondriogene mutations of the types observed have not yet resulted in the occurrence of agents of sufficiently small size to pass the secondary

plasmodesms of graft unions. The agents that have appeared, however, are continuously reproducible in the cytoplasm and produce a type of disease characteristic for virus-infected plants. If the agents concerned became graft-transmissible there would be no question about classifying the abnormalities they cause as "typical" virus diseases. The data thus support previous suggestions that certain plant viruses may have evolved, and are evolving, through chondriogene mutations and natural selection of the progressively more virus-like forms thus produced. The present results show that through the influence (direct or indirect) of nuclear genes the mutation frequency of the chondriogenes may be enormously increased, and that the mutational forms appearing run the whole gamut of those previously presented in a variegational series approaching virus-like forms (*29*).

There appear to be no reasonable grounds for doubting that plant and animal mitochondria are basically homologous cellular organelles—as homologous, *e. g.*, as are plant and animal nuclei. It is believed, therefore, that the results of the present study suggest that a considerable range of pathologic conditions may exist in animal tissues as the result of chondriogene mutations. Such a view has already been expressed (*7*). Technical difficulties, not yet overcome, have made it impossible at the present time to evaluate experimentally the hereditary structure of animal mitochondria. It should be noted, however, that mitochondrial abnormalities, pathologically similar to those caused by chondriogene mutations, may result from the introduction of exogenous viruses into the cell (*31*). These virus-induced neoplasia and their interaction with both normal and mutant chondriogenotypes have been described in the studies mentioned.

SUMMARY

1. The presence of a completely recessive nuclear gene in *Nepeta cataria*, L., was associated with a high rate of chondriogene mutation. All plants homozygous for this gene, *m*, developed mutant chondriogenotypes at one or more places in their soma.

2. The data establish the fact that the chondriome of a single species can undergo extensive mutation, yielding mitochondrial derivatives differing widely in their form and in their action on the cell. The range of variation extends from mutants capable of developing into only slightly abnormal plastids, through intermediate forms which differentiate into strongly modified plastids, to mitochondrial types apparently incapable of developing into plastids at all. Among the latter are certain derivatives that induce cellular abnormalities apparently identical with those produced by infectious viruses in other plants. However, none of these cytoplasmic agents arising in *Nepeta* have been transmissible in grafting or mechanical inoculation tests.

3. Cells with multiple plastid types occurred. The combinations found

correspond to the possibilities for segregation of the mutant mitochondrial types involved.

4. Mutant mitochondria were transmitted unchanged in both sexual and asexual reproduction, the former occurring chiefly through the female gametes. Segregation of chondriogenotypes occurred in both modes of reproduction. Reintroduction caused a reappearance of pathologic symptoms.

5. The mutation-allowing nuclear gene, although requisite for a high rate of chondriogene mutation, was not necessary for survival of the mutant types of mitochondria once these had appeared.

6. It was found, in keeping with other species investigated previously, that chondriogene mutation could occur in the absence of any special mutation-allowing nuclear genes, but only at a very low rate.

7. Pronounced mitochondrial mutation can cause sterility in the plants in which it occurs.

8. Some evidence for the association of chondriogenic mutations is presented.

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PLATE 118

Nine chondriogenotypes in *Nepeta* showing structural appearance (left to right) in physiologically young, intermediate, and mature cells. Semi-diagrammatic. × circa 2,548.

Figure 1.—Normal.
Figure 2.—Light green type 1.
Figure 3.—Macrograna type 1.
Figure 4.—Macrograna type 2.
Figure 5.—Light green type 3.
Figure 6.—Cream.
Figure 7.—White vacuolate type 1.
Figure 8.—White vacuolate type 2 (mitochondria frequently fail to differentiate into plastids).
Figure 9.—Mitochondrial white (mitochondria do not differentiate into plastids).

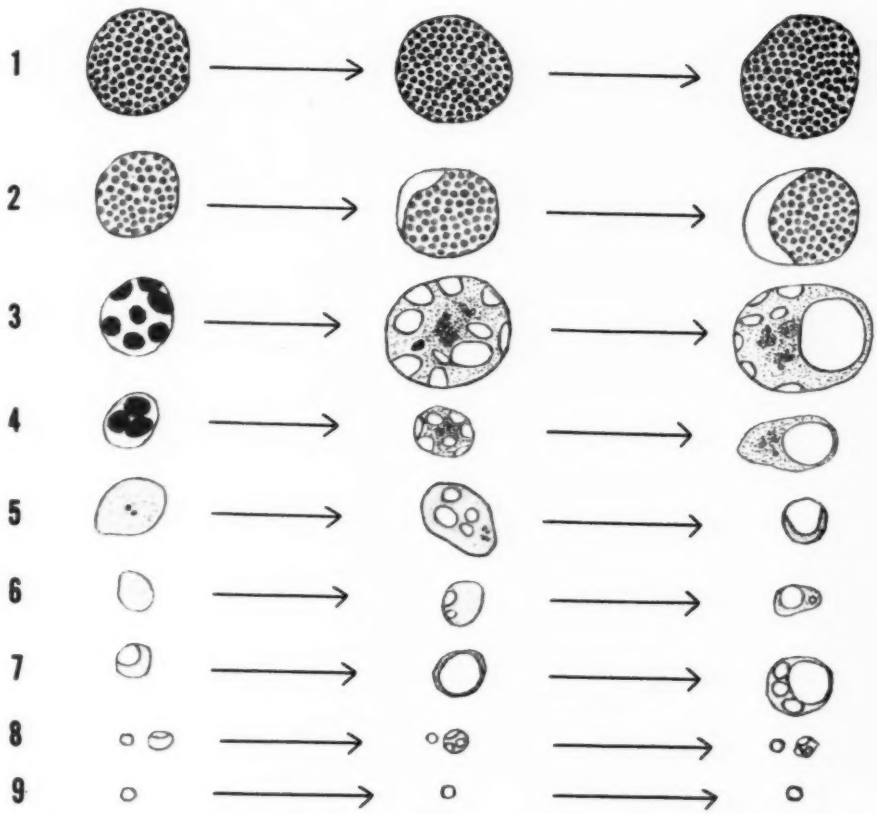

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PLATE 119*

FIGURE 10.—Homochondric normal plant heterozygous for mutation-allowing gene (*Mm*). × ⅔.

FIGURE 11.—Dichondric *Mm* plant with extensive foci of white vacuolate type 1 mutant plastids. × ⅔.

FIGURE 12.—Trichondric *Mm* plant with irregularly distributed foci of macrograna type 1 (light grey areas), and white vacuolate type 1 (white areas) mutant plastids. × ⅔.

FIGURE 13.—Mesophyll of trichondric leaf at juncture of 3 homochondric cell types, white vacuolate type 1 (small colorless plastids), macrograna type 1 (large laterally vacuolate plastids), and normal (lower left).

FIGURE 14.—Portions of 2 dichondric palisade cells containing normal and white vacuolate type 1 plastids.

FIGURE 15.—Dichondric cell with white vacuolate type 1 and macrograna type 1 plastids.

FIGURE 16.—Dichondric cell with normal and macrograna type 1 plastids.

FIGURES 17 and 18.—Two trichondric cells each with normal, white vacuolate type 1, and macrograna type 1 plastids.

*All photomicrographs of living cells × 1,063.

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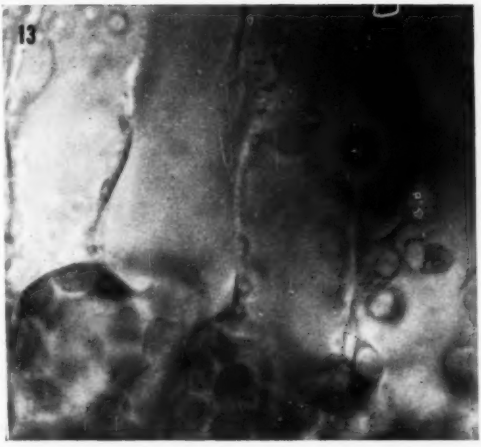
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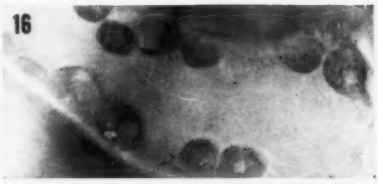
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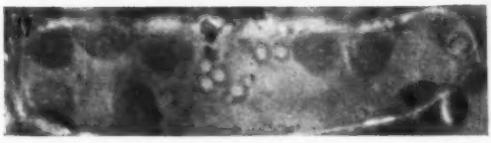

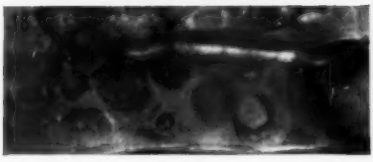

PLATE 120

Seven types of cells from trichondric *Nepeta*. All camera-lucida drawings of living cells × 1,387. Grana not shown in the normal plastids (rendered solid black).

FIGURE 19.—Homochondric normal.
FIGURE 20.—Homochondric macrograna type 1 (vacuolate stage).
FIGURE 21.—Homochondric white vacuolate type 1.
FIGURE 22.—Dichondric, normal, and macrograna type 1.
FIGURE 23.—Dichondric, normal, and white vacuolate type 1.
FIGURE 24.—Dichondric, macrograna type 1, and white vacuolate type 1.
FIGURE 25.—Trichondric, normal, and both mutant types.

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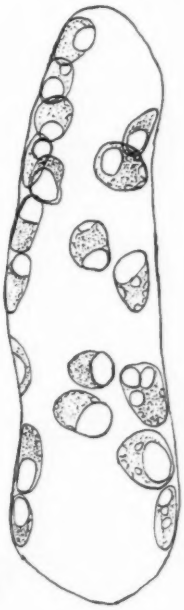

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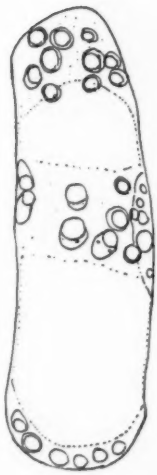

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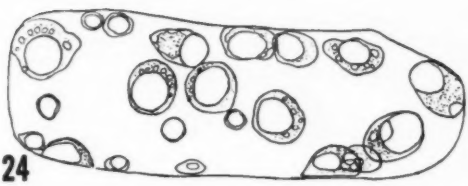

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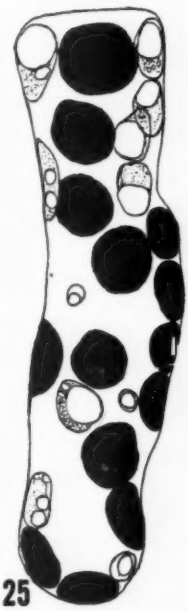

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PLATE 121

Five of the 15 cell types occurring in a tetrachondric *Nepeta* leaf. All camera-lucida drawings of living cells. × 1,544. Grana not shown in the normal plastids (rendered solid black).

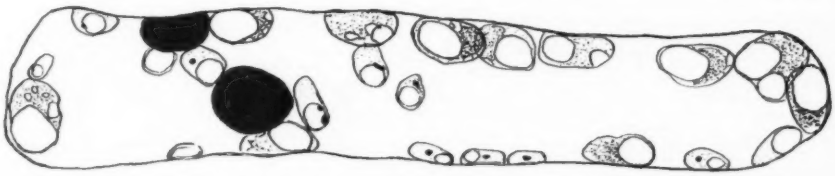
FIGURE 26.—Tetrachondric cell, normal, macrograna type 1 (large vacuolate plastids), light green type 3 (small, often vacuolate), and white vacuolate type 1 plastids (no stippling).

FIGURE 27.—Trichondric cell, normal, macrograna type 1, and white vacuolate type 1 plastids.

FIGURE 28.—Dichondric cell, normal, and light green type 3 plastids (vacuolation beginning in 2 of the mutants).

FIGURE 29.—Trichondric with the 3 mutant types (3 white vacuolate plastids toward center).

FIGURE 30.—Trichondric, normal, macrograna type 1, and light green type 3.



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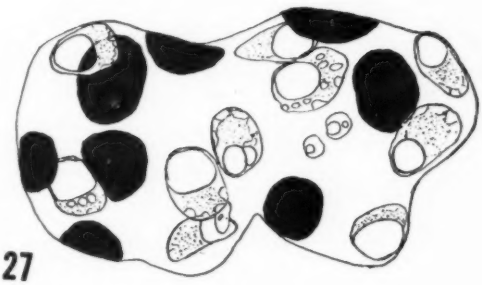

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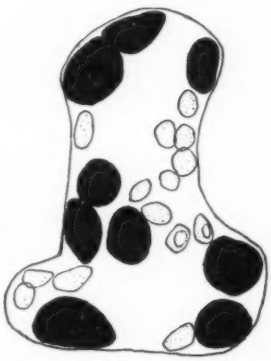

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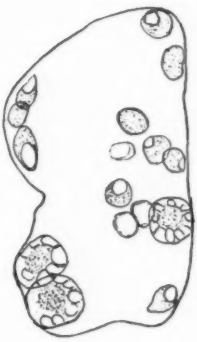

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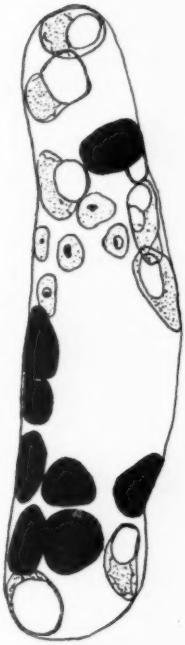

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PLATE 122

Seven different mutant chondriogenotypes in *Nepeta*. All × circa 1.

FIGURE 31.—Light green type 1 showing sectorial segregation of mutant plastids in lower left portion of blade.

FIGURE 32.—Light green type 2 showing mosaic and sectorial segregation.

FIGURE 33.—A type of distorting white mosaic.

FIGURE 34.—Terminal shoot affected with a growth-distorting leaf-curling factor.

FIGURE 35.—Yellow mosaic-producing factor (note similarity to infectious chlorosis illustrated in figure 42).

FIGURE 36.—Green-spotted cream type 1. Dark spots are composed of cells containing nonmutant plastids.

FIGURE 37.—(Figures 37–41 show variable yellow-green type 1 mutant chondriogenotype.) Normal leaf.

FIGURE 38.—Homochondric mutant leaf with predominant yellow symptoms.

FIGURE 39.—Like figure 38, but leaf predominantly green.

FIGURE 40.—Homochondric mutant leaf with green, yellow, and brown necrotic areas. Note vein-yellowing in green apical portion.

FIGURE 41.—Heterochondric leaf showing mosaic segregation pattern of normal and mutant plastids.

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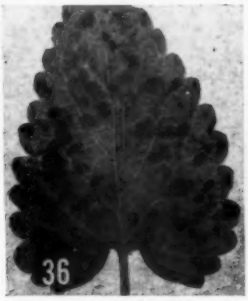
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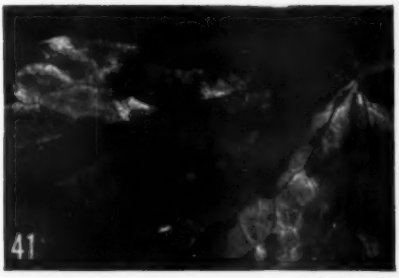
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PLATE 123

Comparison of *Nepeta* leaves infected with an unidentified infectious virus and a leaf containing the noninfectious chondriogene mutant, macrograna type 1.

FIGURE 42.—Heterochondric leaf containing both normal and macrograna type 1 mutant plastids. Note mosaic pattern resulting from plastid segregation. × 2.

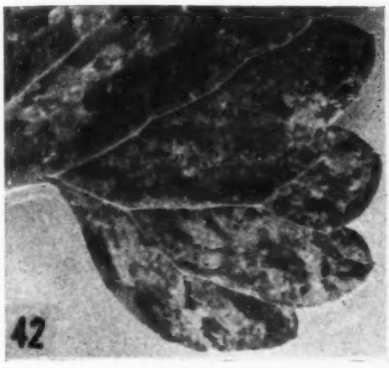
FIGURE 43.—Macrograna type 1 mutant plastids in prevacuolation stage. × 1,740.

FIGURE 44.—Macrograna type 1 plastids at vacuolation stage. × 1,740. Note similarity of figures 43 and 44 to virus-modified plastids in figures 46 and 47.

FIGURE 45.—Two virus-infected leaves. Note relatively diffuse borders (except next to veins) of the chlorotic areas and zonation in leaf at bottom. × 1.

FIGURE 46.—Vacuolation stage of plastids in virus-affected area. × 1,740.

FIGURE 47.—Two plastids in prevacuolation stage in cell of virus-affected area. × 1,740.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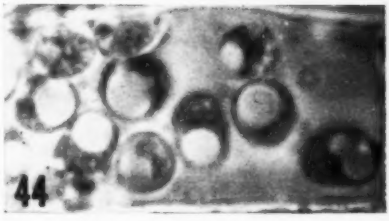
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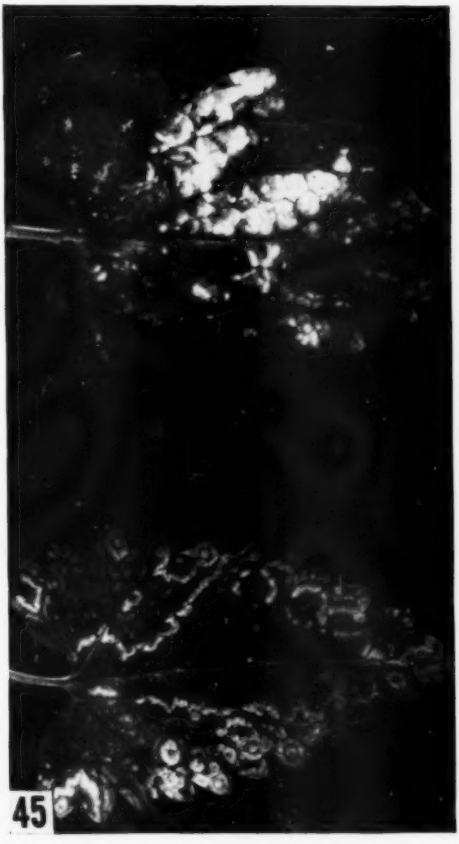
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PLATE 124

Mutant mitochondrial types or derivatives in *Nepeta*. All photomicrographs of living cells. × 1,100.

FIGURE 48.—Normal leaf. × circa ½.

FIGURE 49.—Normal plastids.

FIGURE 50.—Dichondric leaf showing segregation patterns for normal and cream mutant plastids. × circa ½.

FIGURE 51.—Cell homochondric for cream mutant mitochondrial type. Note undifferentiated mitochondria and slightly vacuolate mutant plastids.

FIGURE 52.—Dichondric leaf showing sharp segregation of white vacuolate type 1 mutant mitochondria. Area with colorless mutant plastids shows moderate stunting.

FIGURE 53.—Cell homochondric for white vacuolate type 1 plastids in advanced stage of vacuolation.

FIGURE 54.—Dichondric leaf showing mosaic segregation pattern of white vacuolate type 2 mutant mitochondria. × circa ½.

FIGURE 55.—First layer palisade-cell homochondric for white vacuolate type 2 mitochondria. Note absence of plastids and isodiametric shape of cell.

FIGURE 56.—Similar to figure 40, but vitally stained with Janus green B. Mitochondria dark blue.

FIGURE 57.—Leaf containing partly periclinally localized mitochondrial white mutant mitochondria. Note pronounced inhibition in growth of areas with mutant mitochondria. × circa ½.

FIGURE 58.—Two cells homochondric for mitochondrial white. Mitochondria vitally stained with Janus green B.

FIGURE 59.—Leaf strongly affected with 34–2 type growth-distorting factor. × circa ¾.

FIGURE 60.—Same plant as in figure 44, but whole leaf involved. Note extreme distortion.

FIGURE 61.—Subacute expression of 34–2 growth-distorting factor. × circa ¾.

FIGURE 62.—Cross section of leaf with acute symptoms of 34–2 growth-distorting factor. Whole mesophyll reduced to one cell layer, marked alteration in cell differentiation and reduction in size and number of nonmutant plastids.

FIGURES 63 and 64.—Portion of cytoplasm in 2 cells with acute symptoms of 34–2 growth-distorting factor. Note strong reduction in number and size of nonmutant plastids. Undifferentiated mitochondria stained with Janus green B in figure 64.

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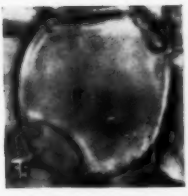

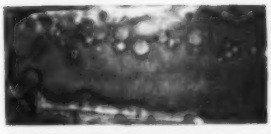

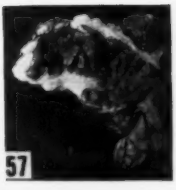
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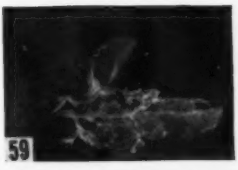
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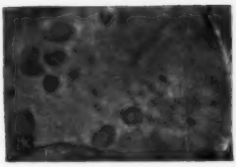


PLATE 125

(Figures 65–70: Supernormal type 1 mutant. Figures 71–73: Three cells from mosaic area in leaf containing white vacuolate type 2 mutant mitochondria. In this case none of the mutant mitochondria had differentiated into plastids. Camera-lucida drawings except for the undifferentiated mitochondria and grana, which were filled in free-hand. × 1,258.)

FIGURE 65.—Young leaf showing focus of stimulated cells that form a blister-like area (enlarged from figure 66). × circa 2.

FIGURE 66.—Leaves showing growth-stimulated areas of darker-than-normal green tissue. Note in particular increase in size of leaf serrations. × circa ¾.

FIGURE 67.—Leaf from old slow-growing branch showing an irregular mosaic of normal, and darker-than-normal green areas. × circa 1.

FIGURE 68.—Palisade cell from normal green area of leaf shown in figure 67. Photomicrograph of living cells. × 1,258.

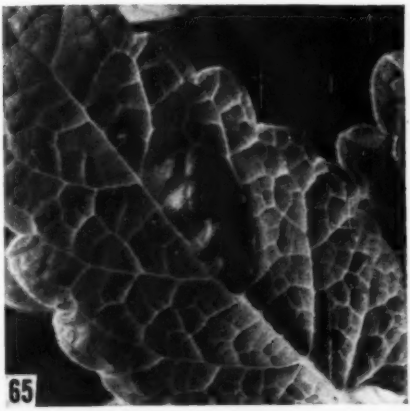
FIGURE 69.—Cell from supernormal area of same leaf. Photomicrograph of living cells. × 1,258.

FIGURE 70.—Cell from mosaic area that has both large and small plastids. Photomicrograph of living cells. × 1,258.

FIGURE 71.—Homochondric normal cell showing plastids and undifferentiated mitochondria.

FIGURE 72.—Heterochondric cell. Note that the nonmutant plastids are reduced in size and that the mutant mitochondria cannot be distinguished from undifferentiated normal mitochondria.

FIGURE 73.—Homochondric mutant cell.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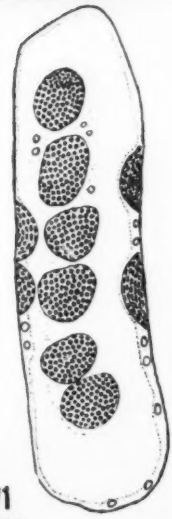
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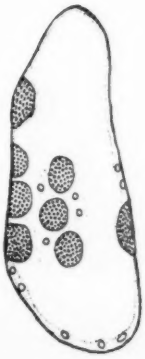
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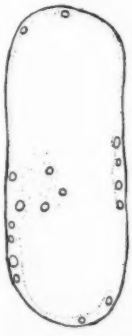
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PLATE 126

FIGURE 74.—Normal clone derived from seedling 35–23.

FIGURE 75.—Clone with growth-distorting factor derived from seedling 35–23. Note malformed leaves and irregular virose-like mosaic of diffuse chlorotic and green areas. × 1.

FIGURES 76–82.—Leaves and flowers from a single polychondric heterozygous (*Mm*) seedling, 140–9. All figures × 1. Figure 76, normal inflorescence. Figure 77, inflorescence from shoot affected with the spider growth-distorting factor. Note the shortened corollas that make the styles and stigmas appear to be abnormally elongated. Figure 78, leaf from one branch that showed the spider type growth-distorting factor. Figure 79, normal leaf. Figure 80, leaf containing normal areas and regions affected with an unidentified stunting factor. Figure 81, leaf with the stunting factor and a type of cream mutant. Figure 82, leaf containing a sectorial area of the cream mutant plastid type (note leaf enlargement) and normal tissue.

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THE EFFECT OF THYROXINE ON THE LYMPHOID-TISSUE MASS OF IMMATURE FEMALE MICE [1] [2]

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In a previous report (*1*), data were presented which indicated that thyroxine treatment of adult male mice was followed by an increase in lymphoid-tissue mass under conditions in which there was not a concomitant increase in rate of growth or a decrease in adrenal cortical function. The present series of experiments in immature female mice was undertaken to study this response in the growing animal and to determine the possible contribution of changes in gonadal function. For the latter purpose also, the effects of thyroxine in adult castrate male mice were observed in a single experiment which is described separately. The results of these investigations allow the statement that the increase in lymphoid-tissue mass which follows thyroxine treatment is independent of both the rate of body growth and the secretory activity of the adrenal glands and gonads.

METHODS

Four separate experiments were performed in order to assay the effect of thyroxine in intact animals (series 1), in animals receiving adrenal cortical extract (series 2), in adrenalectomized animals (series 3), and in animals that had been both adrenalectomized and ovariectomized (series 4). For this purpose, littermate pairs were assigned to one of the four series without plan. One member of each pair was assigned at random to a control group, while its litter mate was assigned to receive thyroxine treatment. Otherwise all the animals in a series were identically (and simultaneously) treated.

Many of the details of the experimental procedures have been previously described (*1*, *2*). Female mice of strain CBA (Strong) were raised on Purina laboratory chow until 5 weeks of age, when they were placed on a diet of bread and milk supplemented by brewers' yeast. At this age also, the operative procedures were carried out. Bilateral adrenalectomy was carried out *via* the lumbar route. The double operation of adrenalectomy and ovariectomy was performed in one stage through the same incision. Adrenalectomized animals were given 0.9 percent NaCl as

[1] Received for publication January 30, 1951.

[2] These studies were carried out in the Department of Radiology, Stanford University School of Medicine, and were supported by a grant to Dr. Henry S. Kaplan from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

[3] While a Postdoctorate Research Fellow and Trainee in Cancer of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

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drinking water throughout the experiment. For the first 3 postoperative days, 5 percent glucose was also added to their drinking water. The adequacy of this dietary regimen for supporting adrenalectomized animals and the evidence for a sustained reduction in adrenal cortical function after operation have been previously detailed (*2*).

Injections of thyroxine and of adrenal cortical extract were begun when the animals were 6 weeks of age and were given daily on 21 consecutive days. Thyroxine [5] was dissolved in slightly alkaline distilled water and a daily dose of 15γ in 0.1 ml. was administered intraperitoneally save for series 4 where a daily dose of 7.5γ was similarly administered. The control solution was prepared exactly as the solvent for the thyroxine, and was given in equal volume. In series 2, a daily dose of 0.05 ml. (2 rat units) of adrenal cortical extract [6] was injected subcutaneously in both groups.

Autopsies were performed 18 hours after the last injections and the various organs dissected and weighed in a routine manner. The sample of lymph nodes consisted of the two axillary nodes on each side and the two epigastric nodes. Operative sites were checked for the completeness of the surgical procedures as well as for the possibility of postoperative infection. All organ weights were determined on a precision torsion balance. Body weights were determined on a double-beam balance at the beginning of treatment and at autopsy.

STATISTICAL CONSIDERATIONS

Statistical methods in common use were employed and $p=<0.01$ was taken as indicating significant differences (*3*). A few points, however, require special comment. Failure to randomize the assignment of the littermate pairs to the several series precludes strict comparison between groups of different series and allows such comparison only between the control and experimental groups of each series.

In series 3 and 4, the intolerance of the adrenalectomized animals to thyroxine (*4*) resulted in a considerable mortality among the animals so treated.[7] The results reported are those of the littermate pairs in which both members survived the experimental period. This ensures comparison of animals which were simultaneously treated and therefore subjected to identical fluctuations of environmental factors and to possible variations in the experimental diet or technical procedures.

The results of all the experiments are recorded as absolute rather than relative (to body weight) values. The differences in body weights observed in series 1 and 2, though significant, are of such small magnitude that adjustment of the organ weights to allow for the body-weight differences does not in any instance alter the significance of the results. Furthermore, analysis of these and other similar data has failed to reveal a significant correlation between body weights and the weights of any of the lymphoid tissues examined.

[5] l-thyroxine, Squibb.

[6] Lipo-Adrenal Cortex, Upjohn.

[7] 11 of 40 adrenalectomized or adrenalectomized and ovariectomized animals died during thyroxine treatment in contrast to the 4 deaths among 40 similarly operated animals that received injections of the control solution.

In contrast, the values of the kidney weights obtained in these experiments showed a highly significant correlation with body weights ($r=+0.7$ to $+0.9$). Adjustment of kidney weights to body weights would eliminate some variation and increase precision but this has not been considered necessary for the present purpose. An analysis of the covariance of kidney and body weights in series 1 and 2 indicates that the increase in kidney weights following thyroxine treatment is greater than would be expected from an increase in body weights alone.

Finally, it should be noted that the experiments are concerned with the dissection and weighing of small organs; and that in some instances the differences described are of such small absolute magnitude that the inaccuracies of measurement approach or exceed the statistical uncertainties. In the case of the adrenal weights, mean and extreme values are presented and the generally accepted principle that differences of 100 percent or greater are significant has been followed. In other instances, the author has been guided by the over-all consistency of the individual determinations.

RESULTS

The results are collected in table 1. Thyroxine treatment of the intact animals of series 1 and 2 was followed by a small but significant increase in the amount of body weight gained. The significance of this difference is reinforced by the facts that in all 28 instances the thyroxine-treated animal gained more than its littermate control, and that this weight gain was registered despite a considerable loss of subcutaneous fat in the thyroxine-treated animals. In contrast, the adrenalectomized animals of series 3 and 4 did not show an increased weight gain following similar treatment.

In all four experiments, the weights of the spleen and lymph nodes were greater in the thyroxine-treated than in the control groups. In series 4, considerable variation was encountered in the splenic weights and the difference is of limited significance ($p=<0.05, =>0.02$). In all other instances the differences are significant. Thus, reduction of adrenal, or of adrenal and ovarian, function did not prevent the response of these lymphoid tissues to thyroxine treatment. Series 2, in which adrenal cortical extract was given to both groups, did not provide significant information probably because the dose of this hormone was too small to have an appreciable effect on splenic and lymph-node tissue.

Histologic examination of representative tissues indicated that the increase in the mass of the spleen and lymph nodes following thyroxine administration was, in large part, due to an increase in the number of all lymphoid elements. While quantitative studies were not performed and an increase in the size of the individual cells cannot be excluded with certainty, the microscopic observations suggested that a true hyperplasia of these lymphoid tissues occurred during thyroxine treatment.

In contrast to the other lymphoid tissues, the thymus was not affected by thyroxine treatment in series 1, 3, and 4. However, when the thymus is under the influence of adrenal cortical hormone, a significant increase in

TABLE 1.—*Body weights and organ weights of immature female mice following treatment with thyroxine and adrenal cortical extract*

Series number	Operation and treatment	Number of mice	Body weights (gm.)			Organ weights (mg.)				
			Initial	Final	Gain	Spleen	Lymph nodes	Thymus	Kidneys	Adrenals [1]
1	Intact, control	16	17.8 ±.3	20.9 ±.3	3.1 ±.2	78 ±3.0	25 ±.7	51 ±1.5	232 ±5.1	5 (3–6)
	Intact, thyroxine [2]	16	17.6 ±.5	22.3 ±.4	4.7 ±.2	140 ±6.5	45 ±1.7	53 ±2.2	327 ±9.2	10 (7–11)
2	Intact, adrenal cortical extract,[3] control	12	17.6 ±.5	21.6 ±.5	4.0 ±.2	80 ±3.3	23 ±.9	33 ±1.9	247 ±5.4	5 (4–7)
	Intact, adrenal cortical extract,[3] thyroxine [2]	12	17.7 ±.7	23.7 ±.5	6.0 ±.2	140 ±6.5	43 ±2.2	40 ±1.8	360 ±6.3	10 (9–12)
3	Adrenalectomy, control	14	17.8 ±.5	21.4 ±.5	3.6 ±.3	166 ±6.5	51 ±2.5	68 ±3.6	232 ±8.9	--------
	Adrenalectomy, thyroxine [2]	14	18.4 ±.5	20.9 ±.6	2.5 ±.3	203 ±9.9	71 ±3.2	69 ±2.4	281 ±11.1	--------
4	Adrenalectomy, ovariectomy, control	13	17.4 ±.4	21.4 ±.5	4.0 ±.3	164 ±5.8	40 ±.8	72 ±2.0	215 ±7.0	--------
	Adrenalectomy, ovariectomy, thyroxine [4]	13	17.3 ±.6	20.1 ±.8	2.8 ±.5	197 ±12.7	50 ±2.1	70 ±2.0	256 ±9.3	--------

[1] Means and extreme range of values. All other values are means and standard error by $\sqrt{\frac{\Sigma d^2}{n-(n1)}}$

[2] 15γ per day for 21 days.
[3] 0.05 ml. Lipo-Adrenal Cortex (Upjohn) per day for 21 days.
[4] 7.5γ per day for 21 days.

its weight follows thyroxine treatment (series 2). Comparison of the control groups of series 1 and 2 indicates that the thymus alone of the lymphoid tissues examined was caused to involute by the dose of adrenal cortical extract employed; and this is in accord with previous studies which have shown the greater sensitivity of the thymus to the action of this hormone (*5*, *6*).

The observations on kidney and adrenal weights indicate that significant increases follow thyroxine treatment. These results confirm previous studies (*7*, *8*) and indicate that these responses are not merely reflections of altered adrenal cortical or ovarian function.

THYROXINE IN ADULT CASTRATE MALES

In order to supplement the results observed in adrenalectomized and ovariectomized mice, the effects of thyroxine in adult castrate male mice have been studied. Male mice aged 12 weeks were castrated *via* the abdominal route. Beginning 1 week later, half of these animals received 21 consecutive daily injections of thyroxine (15γ in 0.1 ml. of slightly alkaline distilled water) while the others received similar injections of a control solution. Autopsies and organ-weight determinations were carried out as in the previous experiments.

These animals are comparable in age and treatment to those of a previous report; and for ease of orientation, the results of the previous study (*1*) are included as groups 1 and 2 in table 2. A significant increase in the weights of the spleen, lymph nodes, kidneys, and adrenals of the castrate animals was observed after thyroxine treatment.

TABLE 2.—*Body weights and organ weights of adult male mice following treatment with thyroxine*

Group number	Operation and treatment	Number of mice	Body weights (gm.)		Organ weights (mg.)				
			Initial	Final	Spleen	Lymph Nodes	Thymus	Kidneys	Adrenals [1]
1 [2]	Intact, control	14	28.3 ±.5	29.7 ±.5	103 ±3.4	32 ±1.2	27 ±.9	470 ±9.6	4 (3–5)
	Intact, thyroxine [3]	14	28.1 ±.5	29.5 ±.5	170 ±8.2	53 ±4.1	22 ±.9	586 ±13.3	5 (4–6)
2 [4]	Adrenalectomy, control	9	27.4 ±.6	28.3 ±.6	161 ±5.3	47 ±1.8	45 ±1.2	441 ±10.8	------
	Adrenalectomy, thyroxine [4]	9	27.3 ±.6	28.2 ±.7	224 ±5.6	61 ±3.2	53 ±3.5	500 ±27.6	------
3	Castration, control	13	------	30.4 ±.6	101 ±7.7	24 ±2.2	45 ±2.2	302 ±6.5	5 (4–8)
	Castration, thyroxine.[3]	13	------	30.1 ±.6	161 ±7.0	36 ±2.4	52 ±2.2	419 ±10.7	9 (6–11)

[1] Means and extreme range of values. All other values are means and standard error by $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[2] These data have been previously published as "Effect of thyroxine on lymphoid tissue mass of adult male mice," Proc. Soc. Exper. Biol. & Med. 72: 42–45, 1949.

[3] 15γ per day for 21 days.

[4] 15γ per day for 14 days.

DISCUSSION

Some of the earliest studies of the effects of thyroid feeding in rats and rabbits elicited suggestive evidence of a stimulating effect of such treatment on the spleen and lymph nodes (*9, 10*). However, recent quantitative studies in rats have led to clearly negative results in this regard (*11, 12*). A likely explanation of the discrepancy between these previous studies in rats and the present observations is the high rate of thyroid activity in the rat. The "resistance" of the rat to thyrotrophic hormone (*13*) as well as evidence from other sources (*14*) indicates that in this animal the thyroid is functioning at nearly maximal levels. Thus the addition of small doses of exogenous hormone may be without detectable effect, while larger doses may lead to "thyrotoxic reactions" of a totally different nature. Meites and Chandrashaker (*14*) have recently reviewed the evidence suggesting a lower level of thyroid activity in the resting mouse, and the present results lend inferential support to such a thesis. If this supposition proves to be correct, it would follow that the mouse is a more suitable animal for studying the metabolic aspects of thyroid function than the more widely employed rat.

Previously, an increase in lymphoid-tissue mass has been observed experimentally only in situations wherein either adrenal or gonadal function has been reduced. Depression of these endocrine functions by thyroxine could explain the increased lymphoid-tissue mass observed in the intact animals of the present experiments. However, the results observed when thyroxine was administered to adrenalectomized and adrenalectomized-ovariectomized females (table 1, series 3 and 4), and to castrate adult males (table 2, group 3) indicate quite clearly that the response of the lymphoid tissues is directly related to the thyroxine treatment and is not merely a reflection of decreased gonadal or adrenal function.

The present observations of an increased rate of growth following thyroxine treatment confirm those of Koger, Hurst, and Turner (*15*) and suggest further that the presence of the adrenal gland is required for this response. The intolerance of adrenalectomized animals to thyroxine makes this finding difficult to evaluate, since failure to gain weight might be simply a reflection of inadequate dietary intake consequent to this treatment. It was observed, however, that the animals that died during treatment with thyroxine did not undergo a prolonged illness but died rather abruptly within 18 to 24 hours of the first outward sign of ill health. The animals surviving treatment were sleek and active and gave no external evidence of ill health. It is therefore suggested that the failure of such animals to exhibit an increased weight gain following thyroxine treatment is directly related to their reduced adrenal cortical function. A more convincing demonstration depends on the possibility of determining a dose of thyroxine which would be adequate for the body-weight response and within the tolerance limits of adrenalectomized animals. Confirmation of the present findings would be of considerable interest, for they suggest a complementary action of adrenal and thyroid hormones.

The response of the thymus gland in the several experiments has been at variance with that of the other lymphoid tissues studied, and a completely consistent explanation of its behavior is not apparent from the data at hand. The increase in adrenal cortical function which follows thyroxine treatment (*16*) and the greater sensitivity of the thymus to the action of this hormone provide the basis of an attractive explanation for the failure of the thymus to show an increase in its mass following thyroxine treatment of intact animals. Thus, the increased level of adrenal cortical hormone would be expected to modify the response of the lymphoid tissues to thyroxine, and the thymus would be expected to show this to a greater extent than the other lymphoid organs. If this explanation were correct, an increase in thymic weight would be expected to follow thyroxine treatment of adrenalectomized animals. This could not be demonstrated, however, as the results in immature females (table 1, series 3) and in mature males (table 2, group 2) indicate.

When the thymus is caused to involute by adrenal cortical hormone, an increase in its mass is seen after thyroxine treatment (table 1, series 2). Thus, under suitable experimental conditions, the thymus may respond to thyroxine in a manner similar to that of the other lymphoid organs. The hormones of the adrenal cortex and the thyroid appear to act independently on this end organ. It is possible that the behavior of the thymus in the other experiments depends upon other hormonal interrelations which remain to be resolved.

The associated increase in the weights of the kidneys and the adrenal glands indicates that the reaction of the lymphoid tissues may be but a part of a generalized response to exogenous thyroxine. Both renal (*7*, *17*) and adrenal (*8*) hypertrophy have been correlated with changes in nitrogen metabolism, and recent studies have shown that lymphoid tissues contribute to the metabolic pool of nitrogen (*18*). Circumstantial evidence would thus point to the role of the lymphoid tissues in protein metabolism as a field for further research.

SUMMARY

The administration of thyroxine to immature female mice was followed by a greater gain in body weight and increased weights of the spleen, lymph nodes, kidneys, and adrenal glands. Following adrenalectomy, similar treatment did not result in a difference in the amount of body weight gained, but the results of the organ-weight determinations were essentially the same. Similar effects on these organ weights were observed following thyroxine treatment of adrenalectomized-ovariectomized young female mice and adult castrate male animals. Thymus weights were not significantly affected in the above experiments. However, when immature female mice received injections of both thyroxine and adrenal cortical extract, thymus weights were greater than in mice receiving adrenal cortical extract alone.

The following tentative conclusions are drawn: 1) An increase of lymphoid-tissue mass follows thyroxine treatment and is not dependent on coincident changes in either gonadal or adrenal cortical function.

2) When thymic involution was brought about by the concurrent administration of adrenal cortical hormone, an increase in the weight of this organ was detectable after thyroxine treatment. 3) The hormones of the thyroid and adrenal glands act independently on the lymphoid end organ. 4) An increased rate of body growth following thyroxine administration may depend upon intact adrenal cortical function.

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STRUCTURAL DETAILS OF SOME OF THE EPITHELIAL CELL TYPES IN THE KIDNEY OF THE MOUSE AS REVEALED BY THE ELECTRON MICROSCOPE [1]

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INTRODUCTION

Previous papers (*1*, *2*) have dealt with some aspects of the submicroscopic structure of certain epithelial cell types in the mouse kidney. The present report is an attempt to describe and summarize in more detail the important structural differences between various epithelial cell types in the kidney of the mouse.

MATERIAL AND METHODS

C3H mice provided the material from which all of the electron micrographs have been taken. A micron marker has been placed on each photograph for convenience in estimating the approximate size of cell components.

Methods of perfusion, fixation, embedding, and sectioning have been described previously (*1*, *2*). The only modification from these techniques has been the substitution of 0.1 percent sodium nitrite for atropine as a smooth-muscle inhibitor, injected intraperitoneally with nembutal in preparation for perfusion. Sodium nitrite affects smooth-muscle fibers directly and acts more satisfactorily than atropine in preventing agonal contraction of the arterioles during perfusion with the fixative.

RESULTS

Sections of the glomerulus and Bowman's capsule [2] reveal that there are three layers interposed between the blood stream and the lumen of Bowman's capsule. The innermost lining is formed by the capillary endothelial cells of which relatively few nuclei (fig. 1) can be seen in a section of any one glomerulus. Frequently the cytoplasm of the endo-

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[2] It is well known that cells similar to those of the proximal convoluted tubule constitute the major portion of the parietal layer of Bowman's capsule in the majority of male mice, whereas in female mice the parietal layer is characteristically made up of squamous epithelial cells. This difference was noted in the present material but no sex differences in structure of the glomerular capillaries or of the visceral layer of Bowman's capsule were observed.

thelial cells is so firmly attached to the underlying basement membrane that the two structures cannot be distinguished from one another. However, where the endothelial cell membranes are clearly visible, they can be seen to possess a fine striation consisting of alternating zones of greater and lesser density (figs. 2 and 3). The almost homogeneous basement membrane forming the intermediate layer is approximately 0.1 μ in thickness. Beyond the basement membrane is an outer layer formed by the fine cytoplasmic processes of the cells of the "visceral" layer of Bowman's capsule (figs. 2 and 3). It should be noted that when a cell process extends for a long distance in contact with the basement membrane (fig. 2), no other processes are visible in this particular area. These cytoplasmic processes in some instances can be seen to form a lattice-like network (fig. 4). They are firmly attached to the basement membrane, and small spaces can be seen between the processes in some areas at the point where they are in contact with the basement membrane (figs. 2 and 3).

In the mouse, the myoepithelioid cells of the juxta-glomerular apparatus have been found to possess rather large cytoplasmic granules (fig. 5). Near its junction with the capillary bed of the glomerulus, the cells form a complete layer around the afferent arteriole (fig. 6).

Membranes of the proximal tubule cells have been shown to be rather prominent in this material under the electron microscope and in sections of cells of the convoluted portion, parts of many cell membranes may be seen in one small field (*1*). Fine, basally located intracellular filament-like structures approximately 0.5 μ in width are also present in the cells of this segment (fig. 7). Cells of the straight portion of the proximal tubule show a simple, rectilinear outline in some instances (fig. 8), but occasionally interdigitation is present between cells in the region near the basement membrane (fig. 9).

A row of vacuoles just under the brush border has been found in cells of the straight portion of the proximal tubule (fig. 8), while such an arrangement of vacuoles has not been found consistently in cells of the convoluted portion. In all instances in which perfusion has been complete, the proximal convoluted tubules have been found to possess a large lumen lined by low "columnar" cells. These cells for the most part contain spherical or short rodlike mitochondria. In the same sections, tubules of the straight segment have a relatively small lumen lined by tall columnar cells in which long filamentous mitochondria are regularly found (fig. 8).

Cells of the thin limb of Henle's loop possess a low striated border consisting of rather widely spaced filaments (*2*). In addition, these cells possess a dense feltwork of fine intracellular filament-like structures oriented vertically along the proximal border of the cell, similar to, but more numerous and obvious than those in proximal tubule cells. Mitochondria do not appear to be associated in any way with these structures (fig. 10). Occasionally, a larger cell type with longer spikelike processes and with a distinctly different internal configuration of the nucleus is found in this part of the nephron (fig. 11).

Cells of the distal convoluted tubule also possess a striated border (*2*) and, in addition, regularly possess intracellular lamellae extending from the basement membrane toward the free surface. Mitochondria appear to be oriented in relation to these lamellae (fig. 12). Osmiophilic droplets, when present in this part of the nephron, have always been found in cells of tubules identifiable by their position as distal convoluted tubules (fig. 13). To date it has not been possible to distinguish cytologically between cells of the thick limb of Henle's loop and cells of the distal convoluted tubule. Distal convoluted tubules were identified by their location near the afferent arteriole of the glomerulus and by the fact that the nuclei of their cells were usually peripherally located (fig. 13). It is assumed that many of the tubules following a straight course in the medullary rays and made up of cells possessing the same cytologic features as the cells of the distal convoluted tubule were in fact tubules of the thick limb of Henle's loop.

Cells of the various levels of the collecting tubules show no essential difference from one another. No specialized surface is visible, and there appears to be no particular orientation of the mitochondria in a cytoplasm which absorbs relatively little of the electron beam (fig. 14).

DISCUSSION

In the past there has been considerable discussion concerning the structure of the glomerulus and Bowman's capsule, particularly in regard to the membrane or membranes involved in the filtration of fluid from the blood stream into the lumen of the capsule. von Möllendorf (*3*) and Bargmann (*4*) described the cells of the visceral layer of Bowman's capsule as possessing numerous processes which, as an interlacing feltwork, form an outer layer covering the basement membrane. They felt that the capillary endothelial cells are similar to endothelial cells located elsewhere in the body and are not especially adapted for the filtration of urine. Bargmann called attention to the fact that while during development the visceral layer of Bowman's capsule is an epithelium, in adult functional kidneys the cells of this layer do not have the appearance of epithelial cells. Rather, they are very similar morphologically to the "pericytes" surrounding capillary endothelial cells in other locations. Volterra (*5*) and Bensley and Bensley (*6*) described argyrophil fibers present in the glomerular lining as being an extension of the fibrous layer surrounding the afferent arterioles. Volterra was of the opinion that these fibers had been confused with cell processes of the cells of the visceral layer of Bowman's capsule. The present material shows clearly that while the structures under discussion have the appearance of fibers, they are in fact processes of the cells of the visceral layer of Bowman's capsule. The processes have a different electron density than the basement membrane. Furthermore, when a process that is obviously continuous with a cell body maintains contact with the basement membrane for some distance, *no other processes are present in this area* (fig. 2). Not all processes are seen to extend from cell body to basement membrane;

however, considering the thinness of the sections and the tortuosity of the processes, it is indeed significant that so many of them were found to be continuous (fig. 3). Examination of a large number of screens also indicates the accuracy of von Möllendorf's observation that the nuclei of the cells of the capsule greatly outnumber the nuclei of the cells of the capillary endothelium. Yet the endothelial cells of the capillaries appear to form a complete lining, while the lining formed by the visceral layer of Bowman's capsule seems to be incomplete. The spaces sometimes seen between the processes at their point of contact with the basement membrane may, however, be artifacts resulting from shrinkage during fixation or embedding. Bensley and Bensley (*6*) found the endothelial lining of the glomerular capillaries to be complete, and, in addition, described a continuous epithelial layer formed by the cells of the visceral layer of Bowman's capsule. They interpreted the cell processes described by von Möllendorf (*3*) and Bargmann (*4*) and shown in more detail in the present paper, as "thickenings in the layer of epithelial protoplasm covering the capillaries." While the present material unquestionably demonstrates the pericyte-like form of the cells of the visceral layer of Bowman's capsule, the possibility still remains that these cells form a continuous covering over the basement membrane. As mentioned previously, spaces appear to be present between the cell processes where they contact the basement membrane. However, it is possible that the cell bodies themselves, from which the processes arise, may form a more superficial but continuous protoplasmic layer. Clear evidence on this point and on the further question as to whether or not this layer is a syncytium has not been found by study of the present material.

The large size of the granules in the cells of the juxta-glomerular apparatus of the mouse kidney, making them visible under the light microscope, suggests that this material should be useful in the study of cytological changes in "polkissen" cells under experimental conditions.

The observations of Grafflin and Foote (*7*, *8*) and Foote and Grafflin (*9*) on the cat and dog kidney have been of value in identifying the various levels of the proximal tubule in the present material. These authors, using silver-impregnation techniques, have confirmed and extended the observations of Schachowa (*10*), R. Heidenhain (*11*), and Zimmerman (*12*) concerning the cytologic differentiation within the proximal tubule. They confirmed the abrupt change in cell contour in the upper part of the straight tubule first observed by Zimmerman. This change is from a complex interdigitation of cell processes in the proximal convoluted tubule to a relatively simple, rectilinear cell outline in the straight tubule. They found the cell contours in the convoluted portion to be more complex near the free surface and near the basement membrane and less complex at the level of the nucleus.

The cell membranes in the mouse, prominent in this material under the electron microscope, show complex interdigitations in the proximal convoluted tubule but are primarily simple and rectilinear in the straight

tubule. In sections of this type it obviously could not be determined whether or not this transition in cell contour is abrupt.

When the numerous interdigitating membranes of the cells of the proximal convoluted tubules were first observed in this material, some of them were mistakenly identified as intracellular fibrils. However, it was found that true cell membranes are always either continuous from basement membrane to free surface or are continuous as a loop returning to the surface of origin. Differentiations of filament-like strands are present in the cytoplasm of the proximal tubule cells but they have been found only in the basal parts of the cells and are somewhat thinner (approximately 0.05 μ) than cell membranes. They are also identifiable by the fact that they terminate in the cytoplasm without returning to one of the tubule surfaces.

The filament-like structures visualized in cells of the proximal tubule and in the thin limb of Henle's loop and the distal convoluted tubule are interpreted as representing areas of cytoplasm possessing greater opacity to the electron beam than the adjacent cytoplasm. These structures are, as a rule, oriented in a position perpendicular to the basement membrane. An attempt has been made to determine whether they are filaments or actually lamellae. The great depth of focus of the electron microscope makes it difficult to answer such a question simply by examining them while varying the focus. Electron micrographs of the different levels of the nephron, however, show that these filament-like structures frequently remain in sharp focus while adjacent mitochondria with a much greater diameter go in and out of focus. This is particularly obvious in the distal convoluted tubule. It is suggested therefore that in the distal convoluted tubule at least, the structures that appear to be intracellular fibrils are actually lamellar in form. While Mislawsky (*13*) and M. Heidenhain (*14*) inferred from the intracellular organization the existence of intracellular fibrils or lamellae in the cells of the proximal tubules, they probably did not see the structures (0.05 μ thick) described in the present material. Mislawsky included a diagram in his paper which presented his interpretation of the structure of the cells of the convoluted tubule. In this diagram he illustrated vertically oriented lamellae, assumed to be present, and responsible for the vertical orientation of mitochondria in these cells. Such structures are in fact present in the distal convoluted tubule but as indicated above, their diameter is such as to preclude their being individually resolved by the light microscope. There is the possibility, however, that an absorption of stain on the surface of such lamellae could thus make them visible.

It is possible that the same cellular activity responsible for the presence and orientation of these structures is responsible for the similar orientation of the mitochondria in the various parts of the nephron. However, it should be pointed out that the thin limb of Henle's loop, in which they are particularly prominent in the proximal part of the cell, does not show the clear-cut orientation of the mitochondria characteristic of the cells of the proximal and distal convoluted tubules.

SUMMARY

The majority of the epithelial-cell types of the mouse kidney have been examined under the electron microscope.

Myoepithelial or "polkissen" cells with large cytoplasmic granules have been observed.

Three membranes are considered to be interposed between the blood stream and the lumen of Bowman's capsule—a complete endothelial lining, a continuous basement membrane, and a layer formed by the fine interdigitating processes of the cells of the visceral layer of Bowman's capsule.

The complex interdigitation of cell boundaries of proximal convoluted-tubule cells is clearly evidenced in this material by the resolution of the tortuous cell membranes.

A low "striated" border and basally located, vertically oriented intracellular fibrils or lamellae are characteristic of the majority of cells of the thin limb of Henle's loop. Larger cells with spikelike processes are sometimes found in this part of the tubule.

A low "striated" border on the free surface and vertically oriented intracellular lamellae extending from the basement membrane, almost to the free surface, are characteristic of cells of the distal convoluted tubule.

Collecting tubule cells show no surface specialization.

ADDENDUM

Pease and Baker (*15*) recently presented some observations on various cells of the kidney of the rat as viewed under the electron microscope. Their report appeared after the present paper had been submitted for publication, and contains several differences in results and interpretation of results from those presented above. The first and probably greatest important difference is concerned with fixation. Pease and Baker found that "kidney tissue fixed without perfusion appeared as well preserved as the very best of the perfused material." Experience in this laboratory has been just the reverse of this in that fixation by perfusion, particularly with a fixative containing osmic acid, has always been found to be superior to fixation by immersion. This finding is probably correlated with the observation that osmic acid penetrates kidney and liver tissue at the rate of only 0.5 mm. per hour.

This fundamental difference in technical method probably accounts for the other differences in results, notably in connection with the presence or absence of an endothelial lining in the glomerular capillaries, with the point of origin of the processes covering the basement membrane of the glomerular capillaries, with the presence or absence of vacuolated mitochondria (not found in the present material), and with the presence or absence of filamentous projections from the surface of cells of the distal convoluted tubule.

It is felt that it would be relevant at this point to comment on the general problem of the interpretation of cell structure as visualized by the electron

microscope. As with phase-contrast microscopy, structures observed under the electron microscope must be interpreted in relation to the visualizing source—in the case of the electron microscope—the electron beam. It is reasonable to assume that structures with different electron densities are different from one another, either physically or chemically. On the other hand, it cannot be safely assumed that two structures with the same electron density are identical in either of these respects. With the difficulties of interpretation in mind, it can safely be said that, as in the case of the classical methods of cytology, real advances in our knowledge of cell structure from the study of tissues under the electron microscope can come only after critical evaluation, standardization, and proper utilization of technical methods.

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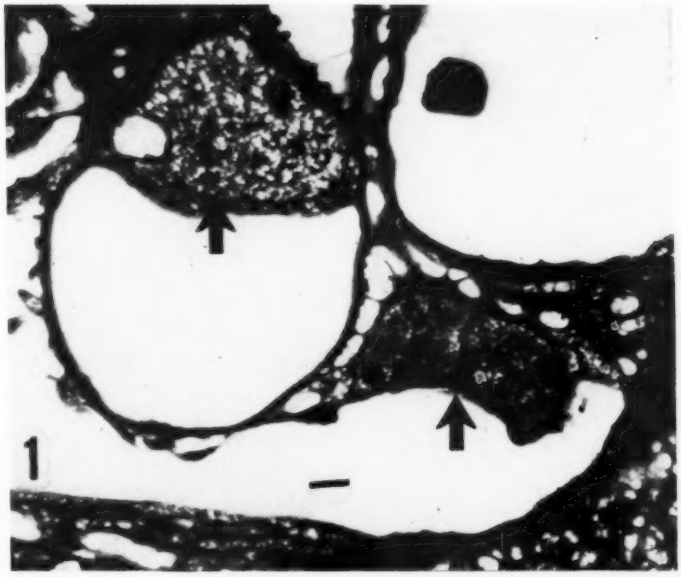
PLATE 127

FIGURE 1.—Part of a glomerulus showing a nucleus of an endothelial cell (upper arrow) and a nucleus of a cell of the visceral layer of Bowman's capsule (lower arrow). Details of the endothelial and capsular lining are not evident. × 5,000.

FIGURE 2.—Part of a glomerulus showing the processes of cells of Bowman's capsule (one at lower arrow), some lying against and others extending to the basement membrane. The upper arrow and the micron marker are each in the lumen of a capillary. The lower arrow lies in the space of Bowman's capsule. Fine striations in the endothelial membrane are present (upper arrow). × 10,000.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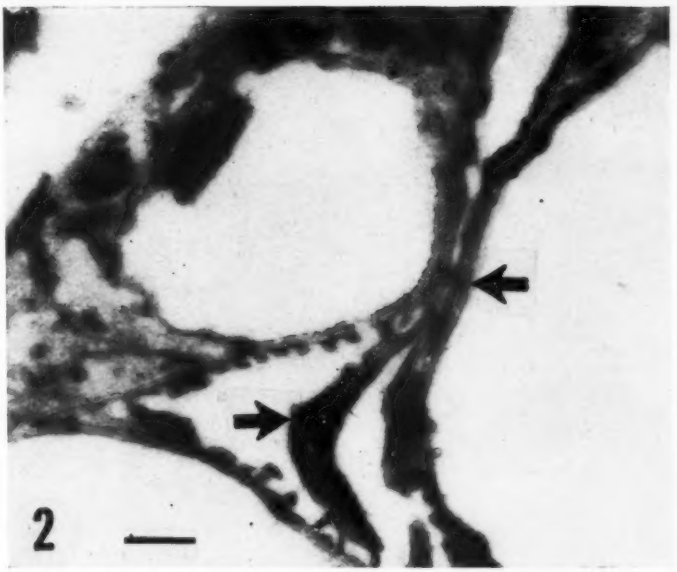
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PLATE 128

FIGURE 3.—Part of a glomerulus showing two cells of Bowman's capsule (left center and center) and parts of two glomerular capillaries (upper left and right). The processes of the cells extend in several directions and rest against the basement membrane covering the endothelial lining. The spaces between the cell bodies and the basement membrane possibly were formed during the perfusion procedure. Open spaces are present between some of the processes (arrow). × 8,000.

FIGURE 4.—A tangential, thick section through parts of cell processes and cells of the visceral layer of Bowman's capsule at a level just beyond the basement membrane of a capillary. The processes of the cells are fiber-like in appearance and apparently interdigitate with one another (arrow). × 12,000.

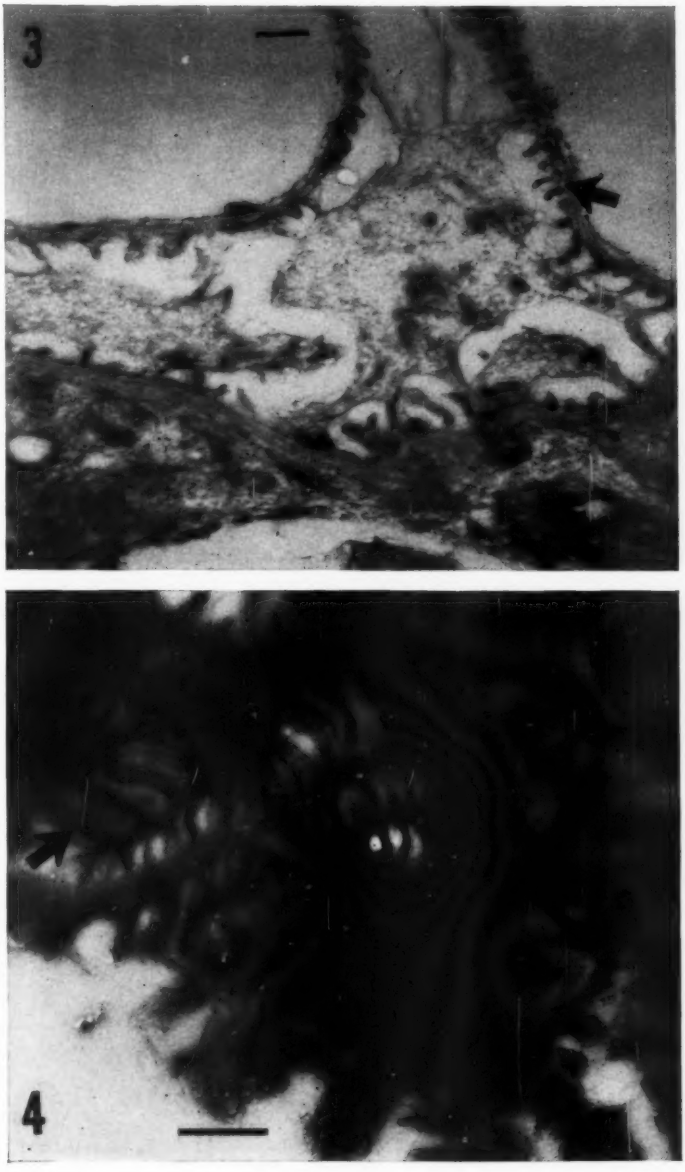
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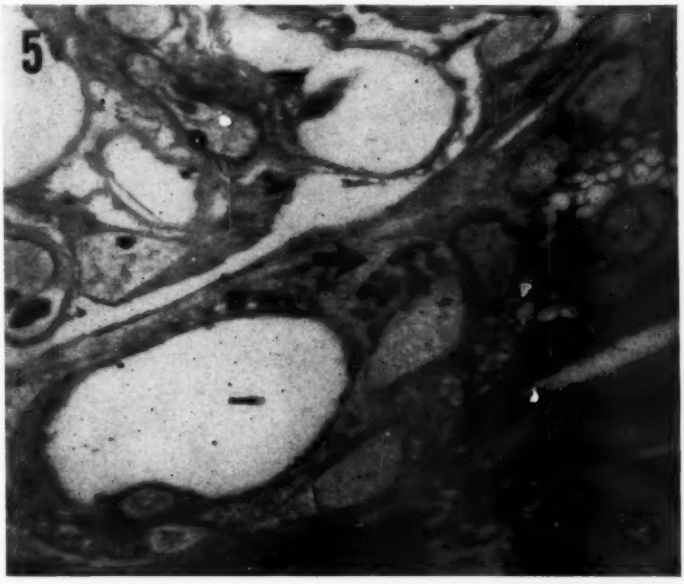
PLATE 129

FIGURE 5.—An afferent arteriole of a glomerulus showing a myoepithelial "polkissen" cell with cytoplasmic granules (arrow). × 3,000.

FIGURE 6.—Part of an afferent arteriole of a glomerulus with a continuous covering of "polkissen" cells containing large cytoplasmic granules. A cell membrane is evident (arrow). × 8,000.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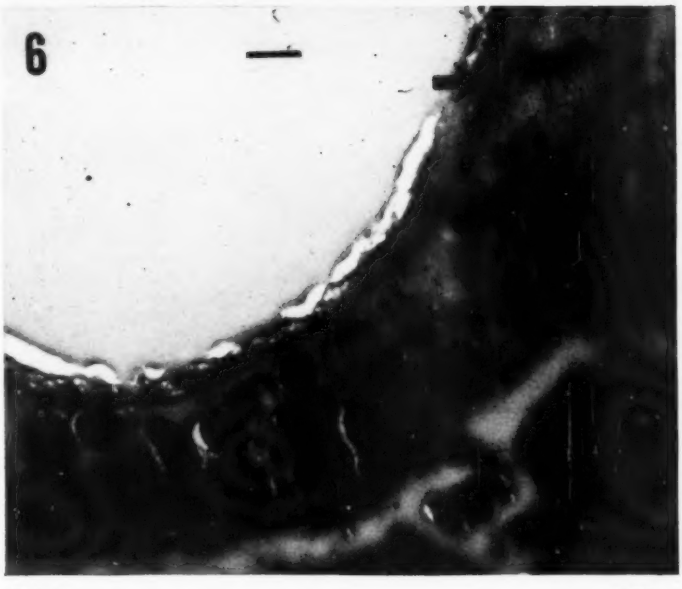
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PLATE 130

FIGURE 7.—Part of a cell of the proximal convoluted tubule showing tortuous cell membranes (upper arrow) and basal intracellular "filaments" (lower arrow). × 8,000.

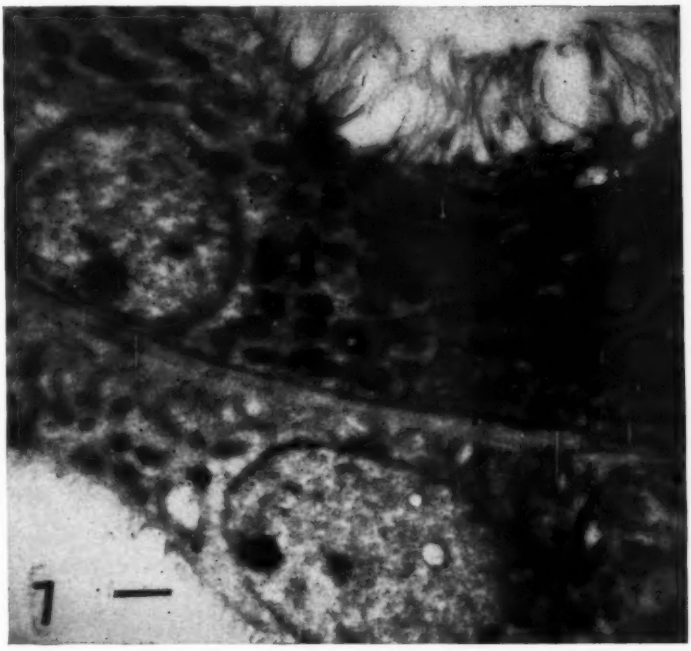
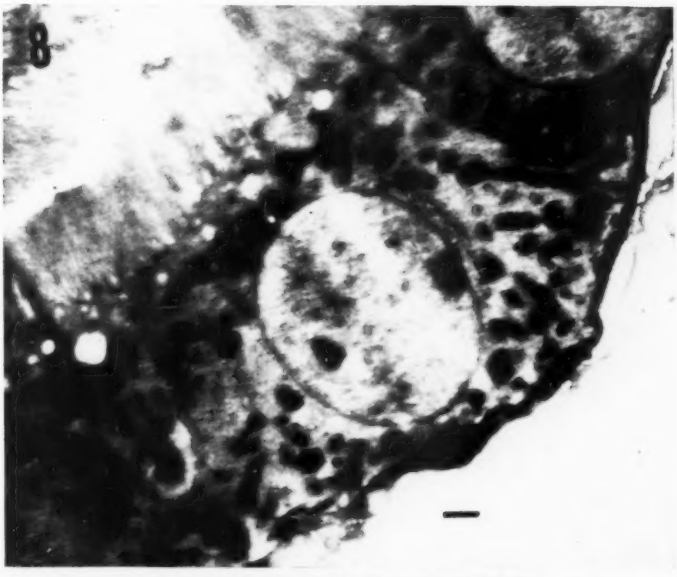


PLATE 131

FIGURE 8.—A cell of the straight portion of the proximal tubule containing filamentous mitochondria and possessing a rectilinear outline. × 5,000.

FIGURE 9.—A cell of the straight portion of the proximal tubule interdigitating cell membranes at the basal end of the cell (arrow). × 5,000.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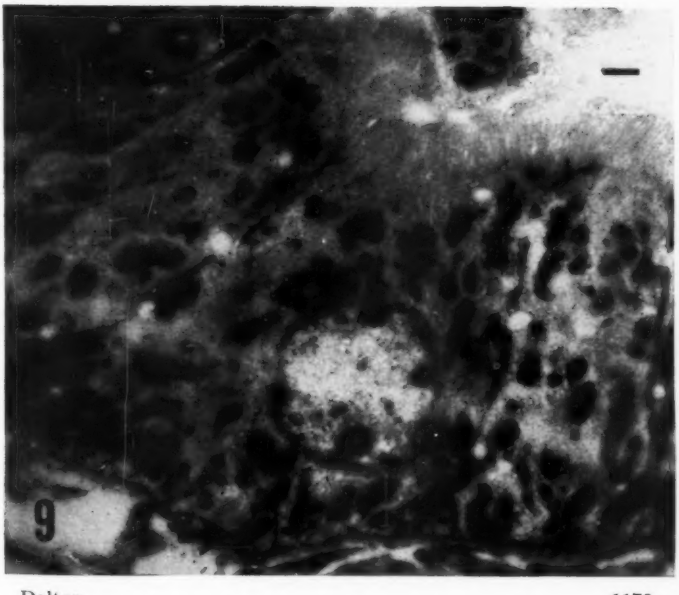
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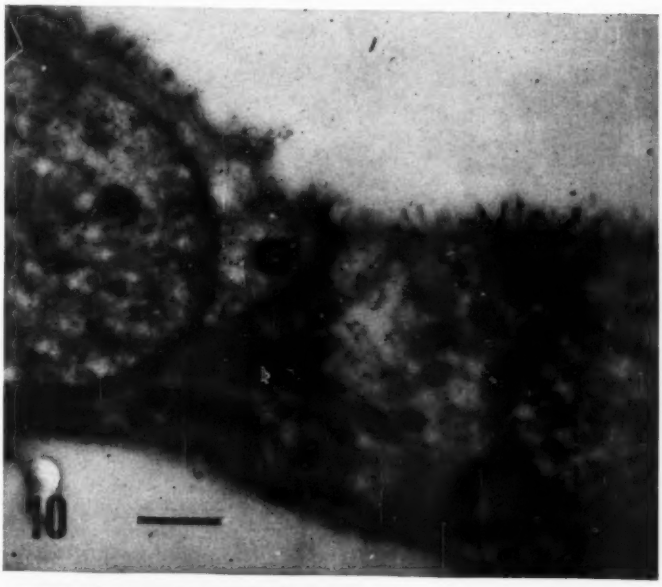
PLATE 132

FIGURE 10.—Parts of two cells of the thin limb of Henle's loop showing numerous intracellular basal "filaments" and a "striated" border. × 12,000.

FIGURE 11.—A longitudinal section of a thin limb of Henle's loop showing the usual cell type (right) and a larger cell (left) with a different type nucleus and longer spike-like processes at the free surface. × 8,000.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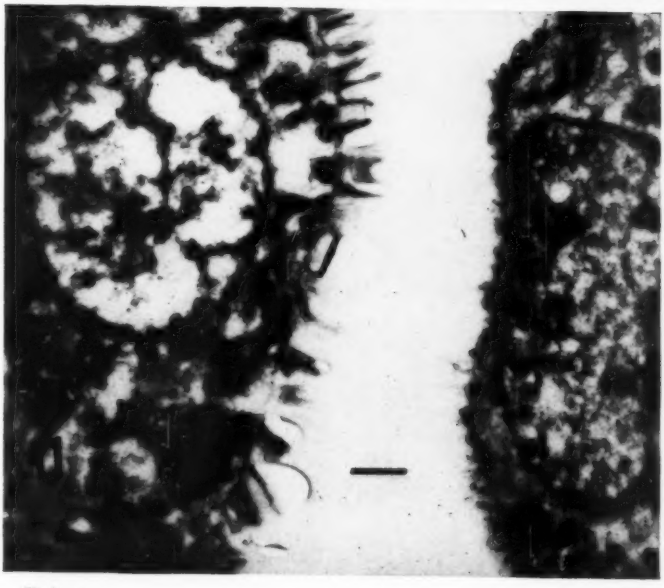

PLATE 133

FIGURE 12.—Part of a cell of a distal convoluted tubule with a "striated" border and vertically oriented intracellular lamellae. × 12,000.

FIGURE 13.—A cross section of a distal convoluted tubule showing filamentous mitochondria, peripherally located nuclei and osmiophilic droplets in the peripheral cytoplasm. × 3,000.

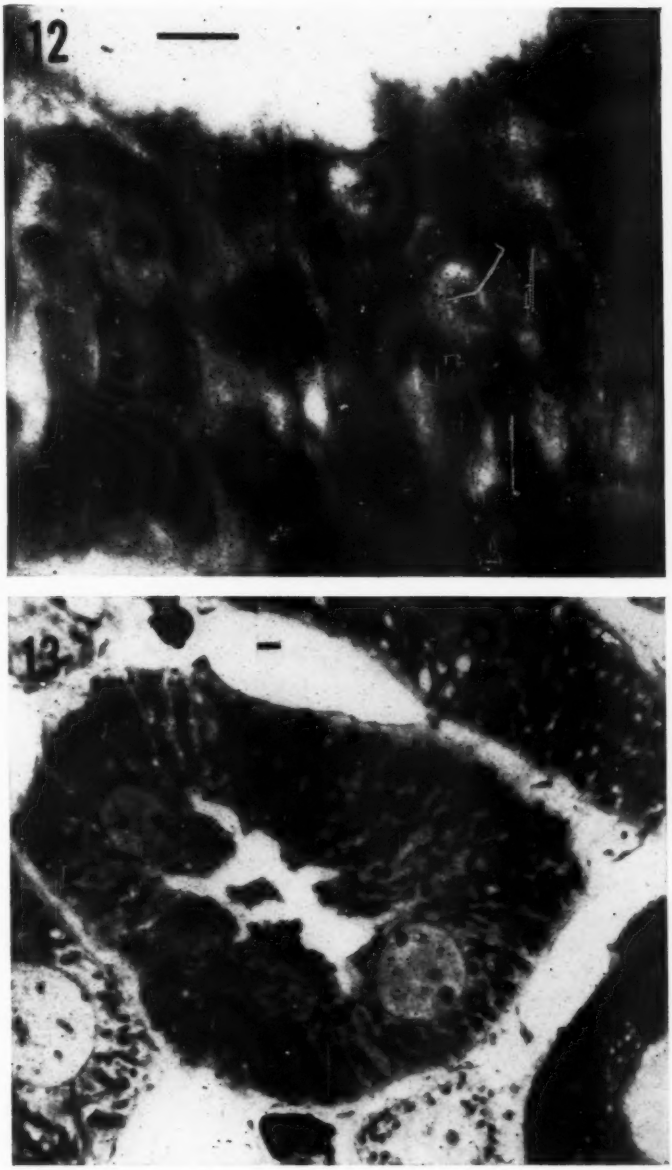
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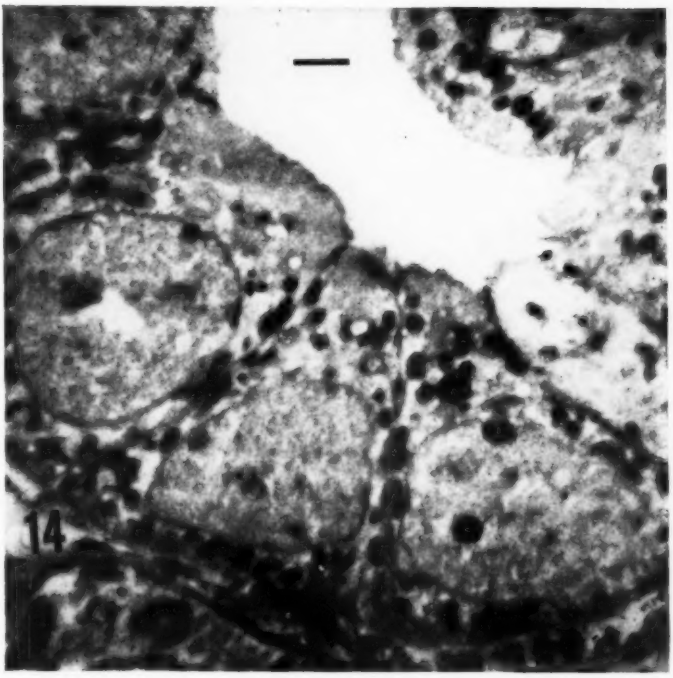
PLATE 134

FIGURE 14.—Cells of a collecting tubule showing an undifferentiated free surface. × 8,000.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THE HISTOPATHOGENESIS OF PULMONARY TUMORS INDUCED IN STRAIN A MICE BY URETHAN [1] [2]

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An experiment was undertaken to determine the site of origin of urethan-induced pulmonary tumors in strain A mice (*1*). The urethan-induced pulmonary tumors were examined not only in the later stages but also during the developmental period. Particular attention was paid to the relationship of these tumors to bronchioles and alveoli and to the existence of inflammatory and related conditions such as lymphoid infiltration, atelectasis, tracheobronchitis, pneumonitis, granuloma, and fibrosis.

EXPERIMENTAL OBSERVATIONS

Methods

Two hundred strain A mice, 2½ months of age, were separated into 2 groups, each equally divided as to sex. One group was given 0.1 percent solution of urethan in drinking water for 13 weeks, while the other group received the same diet and drinking water without urethan. At the end of the first week and each week thereafter, a minimum of 6 treated and 6 control animals were examined post mortem. All the animals were killed by ether anesthesia, and the lungs were fixed by intratracheal injection of 0.5 cc. of Zenker's acetic-acid fixative. Occasionally a lung was fixed in formalin. Routine sections were taken from all the organs and from at least 3 lobes of the lungs of every animal. In addition to this, 1 lobe of the lung from 2 animals treated with urethan for 1, 6, and 11 weeks, respectively, and 1 lobe of the lung from each animal treated for 2, 3, 4, 5, 7, 8, 9, 10, 12, and 13 weeks, respectively, and 1 lobe from each of 3 control animals, 1, 2, and 4 weeks, respectively, after the initiation of the experiment, were examined in serial sections. Most of the sections were stained by hematoxylin and eosin, a few by Verhoeff's elastic-tissue stain, and an occasional one by Wilder's silver-impregnation method for reticulum and by the periodic-acid technique.

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[2] Presented in part before the American Association for Cancer Research, Atlantic City, New Jersey, March 1948.

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General Considerations

All the animals treated with urethan for 6 weeks or longer showed multiple lung tumors on gross examination, whereas no lung tumors were seen grossly in the controls.

In general, there was striking similarity among the lungs of the experimental animals in any given period. Those of the control animals also resembled one another.

On the basis of the experimental evidence to be presented, we believe that urethan-induced pulmonary tumors arise from certain cells that occur normally in the mouse lungs. In the lungs of untreated mice these cells, which are found singly or in groups of two or three lying in the angles of the alveoli and sometimes appearing to cover the alveolar walls, are usually referred to as septal cells and less frequently as alveolar cells. In the present communication we have used the term alveolar cells in referring to them.

Histopathology of Lungs of Treated Animals

First 3 weeks. The lungs of the experimental animals autopsied at the end of the first week showed no increase in the incidence of pneumonia, atelectasis, lipophagic or inflammatory cell infiltration of the alveoli, edema, or desquamation of bronchial cells as compared with the controls. Two lobes of one animal showed patchy atelectasis. In three lobes of another animal there was peribronchial lymphoid infiltration; in one of these, atelectasis was also present. The lungs of one of the control animals showed peribronchial lymphoid infiltration, focal atelectasis and early pneumonia in all the lobes. Early pneumonia without atelectasis was seen in three and five lobes, respectively, of two other control animals. The alveolar cells in some of the experimental mice were slightly more prominent, and there was a suggestion of increased cellularity (fig. 1).

By the second, and especially by the third week, the lungs of the experimental animals could be differentiated from those of the controls after close comparison. The alveolar cells, particularly in focal perivascular and subpleural areas, were increased in number (figs. 2, 3, and 4). The cells were large, measuring from 15 to 20 microns in diameter. The nuclei were large, hyperchromatic, and contained one or two nucleoli. Binucleated cells were not infrequent, and occasionally a group of two to five nuclei, apparently in a single mass of cytoplasm could be seen close to one another in the angles of some of the alveoli. The exact site of these cells was difficult to determine. They sometimes bordered the walls of the alveoli, often gave the appearance of an alveolar lining, sometimes filling the alveolar lumen.

From the start of the second week of urethan treatment, a few mitotic figures were seen in cellular areas similar to those described for the first week, and more of them appeared in the third and succeeding weeks. The mitotic activity appeared to involve alveolar cells rather than vascular endothelial lining cells. Rarely a mitotic figure was seen in apparently

detached cells, some of which we believe represented alveolar cells protruding into the alveolar spaces, as evidenced by examination of serial sections. The elastica of the alveolar walls appeared normal.

In one lobe of an animal treated with urethan for 2 weeks there was an area of fibrosis and chronic inflammation. In another animal peribronchial lymphoid infiltration and some atelectasis were seen in all the lobes. In a third animal all lobes showed early pneumonia. Two lobes of the lung of one of the control animals examined at the end of the second week were atelectatic; in all the lobes of another control animal pneumonic consolidation was present while in one lobe of two other animals there was an area of fibrosis and chronic inflammatory-cell reaction.

In one lobe of an animal treated for 3 weeks atelectasis and early pneumonia were noted. In another lobe of this animal and in a lobe from another animal a small area of granulomatous reaction was found. A similar area was encountered in a lobe from one of the control animals examined at the end of the third week.

In all the other animals, both experimental and control, all the air passages were open and the lungs were free from any inflammatory reaction. The bronchiolar epithelial lining was intact.

Fourth and fifth weeks. Grossly, the lungs of treated animals showed no significant change. Microscopically, as compared with the controls, the lungs were definitely more cellular. The proliferated alveolar cells were seen bordering part or all of the walls of several (2 to 10) adjacent alveoli or alveolar sacs (fig. 5). Although a few of these involved alveoli were centrally located, most of them were situated subpleurally and were not related to any bronchus.

A finding that characterized these lungs and differentiated them from the controls and from the lungs of the experimental animals examined prior to the fourth week was the increase in number of mitotic figures. As compared to the controls, the lungs of treated animals showed an increase in mitoses, restricted almost entirely to the alveolar cells (fig. 6). Although no mitotic counts were made, evidence for this increase was based on the fact that only a rare histologic section of a control lung contained even a single mitotic figure, while nearly every section of lung of the urethan-treated animals showed 5, 10, or more mitotic figures.

In the sections of a few of the lungs from mice treated with urethan for 5 weeks, an unusually large cell was observed in the angles of the alveoli. Its giant nucleus was hyperchromatic and in some cases showed considerable variation in size and shape and contained acidophilic or unstained inclusion bodies. The cytoplasm was acidophilic. These cells were seen more frequently in the succeeding weeks of treatment.

None of the six animals treated with urethan for 4 weeks showed any evidence of inflammatory reaction. Peribronchial lymphoid infiltration and a small area of fibrosis were found in one lobe of one of the control animals.

Two lobes of one of the animals treated for 5 weeks, and one lobe of another experimental animal showed diffuse pneumonic consolidation

while in one lobe of a third animal there was a large area of fibrosis. Focal pneumonia and consolidation were found involving all the lobes of two control animals examined at the same time. There was early pneumonia in five and four lobes, respectively, of two other control animals.

Sixth week. In one animal of this group, there were two grossly visible subpleural lung tumors, each measuring about 1 mm. in diameter. One of these is shown in figure 7.

In all the other lungs, groups of adjacent alveoli were lined by cuboidal cells, filling the spaces almost completely in some instances (figs. 8 and 9). Many mitotic figures were found in these cells. These adenomatoid areas were also usually subpleural or, if deep in the lung, were rarely close to a bronchus. The alveolar architecture was clearly maintained. Three lobes of one of the animals treated for 6 weeks showed pneumonic consolidation, which was also seen in two lobes of one of the control animals. Another control animal examined at 6 weeks showed congestion, atelectasis, and early pneumonia in one lobe. Inflammation and atelectasis were otherwise absent. The bronchi were perfectly normal.

Seventh week. Microscopically demonstrable tumors were present in all lobes. The tumor tissue consisted of cuboidal cells closely packed, with very little stroma. At the periphery of the tumor the alveolar architecture was retained, although the alveoli themselves were lined with cuboidal alveolar cells. Elsewhere in the lungs, groups of 5 to 20 adjacent alveoli were lined by similar cells. Mitoses were abundant in these areas.

The intervening lung tissue was essentially normal, except that an occasional alveolus contained the large hyperchromatic cells which have been described above. Similar cells were sometimes seen in the tumors.

In two lobes of one of the animals treated for 7 weeks there were focal pneumonia and granuloma formation. Four lobes of one of the control animals showed early peumonia while in two lobes of another animal there was atelectasis with focal granulomatous reaction and fibrosis. Atelectasis was also seen in a lobe of a third control animal examined at the end of the seventh week.

Eighth, ninth, and tenth weeks. The lungs of all the experimental animals in these groups contained tumors in various stages of development, the largest measuring about 1 mm. in diameter (fig. 10). The histologic pattern of the lung tumors was similar to that of tumors found in the earlier and succeeding weeks and is described in detail later in this paper.

The alveolar walls of the intervening lung tissue showed scattered foci displaying increased cellularity with hyperchromatic cells and many mitotic figures in the alveolar cells. This gave a histologic picture quite like that seen in the fourth and fifth weeks.

Two lobes of one of the animals treated for 8 weeks were involved in pneumonic consolidation. One pulmonary lobe of another animal was atelectatic and had peribronchial lymphoid infiltration. In one lobe of a third animal an area of fibrosis was present. Four control animals examined at the end of the eighth week showed evidence of pulmonary

inflammatory reaction: pneumonic consolidation was present in all lobes of one animal. Lymphoid infiltration and congestion were seen in one lobe of each of three animals. In two of these lobes also there was an area of fibrosis.

One of these animals treated with urethan for 9 weeks and a control animal of the same age examined at the same time showed pneumonic consolidation of all the lobes. Two other control animals showed early and focal pneumonia in four lobes.

Three animals treated with urethan for 10 weeks had pneumonic consolidation in all the lobes. One of the control animals examined at the end of 10 weeks also showed pneumonia in two lobes, while three other animals had early pneumonia in five, four, and two lobes, respectively.

Eleventh, twelfth, and thirteenth weeks. Tumors 1 or 2 mm. in diameter were seen in the lungs of all the treated animals. They were pearly white, slightly bulging, and spherical, and situated chiefly in the periphery of the lung.

Microscopically the tumors resembled those described by previous investigators. Most of the cells composing the tumor were cuboidal or polygonal. The cytoplasm was pale, acidophilic, and frequently finely foamy. The nuclei were large, oval or spherical, vesicular, and possessed one, and sometimes two, nucleoli. Mitotic figures, binucleated cells, and giant cells with hyperchromatic nuclei were less commonly present than was the case in the previous weeks. The cells formed acini, lined structures which appeared to be preexisting alveoli, or occurred as narrow sheets or columns, which in some cases surrounded the alveolar ducts. Alveolar macrophages and cellular debris were observed in a few of the acinar spaces. Polymorphonuclear leukocytes were rarely found. There was little if any evidence of compression or fibrosis of the surrounding lung tissue.

In about 20 percent of the cases, the tumors were partly or largely composed of columnar cells presenting a papillary acinous pattern. The stroma was scant and consisted of a well vascularized fibrous tissue with a few fine elastic fibers.

The tumors were not encapsulated. Instead, the alveoli at the periphery of the tumor were frequently lined by cuboidal alveolar cells (fig. 11) suggesting that the increase in size of the tumor was due, in part at least, to the continued neoplastic development of new tumors in the alveoli at the periphery of each tumor. As successive serial sections of the lungs of animals treated for 8 to 13 weeks were studied, it was at once evident that the histologic picture seen in the sections obtained from the peripheral portions of a fully developed tumor was quite similar to that seen in the lungs of mice treated for shorter periods of time (*cf.* figs. 13, 14, and 15 with figs. 4, 5, and 9, respectively).

Foci of greater cellularity and of increased numbers of mitoses were especially prominent in the intervening lung tissue. In many of these areas were giant cells, measuring 20 to 30 microns in diameter, with large hyperchromatic nuclei and occasional large clear or acidophilic nuclear

inclusions (fig. 12). Many of these foci were not topographically or histologically related to any of the tumors, and, viewed separately, were similar to areas seen in the lungs of animals treated for shorter periods of time.

Evidence of pulmonary inflammation was seen in only a few animals. One animal killed at the end of the eleventh week of urethan administration had pneumonic consolidation in all the lobes; while another animal showed this process in only one lobe. Early pneumonia was seen in one lobe of another animal treated for 11 weeks. In four control animals examined at the same time pneumonic consolidation was found in five, four, three, and two lobes, respectively, while in a fifth control animal there was early pneumonia in all the five lobes. Two of the animals treated for 12 weeks had pneumonic consolidation in all the lobes. In another animal peribronchial lymphoid infiltration and tracheobronchitis were present in one lobe. Three of the control animals also showed complete pneumonic consolidation of all the lobes. One of the animals treated with urethan for 13 weeks had pneumonic consolidation in all five lobes. This was also seen in two of the control animals killed at the same time. Two other control animals also showed pneumonic consolidation involving two lobes and one lobe, respectively.

Histology of Lungs of Control Animals

Since 17 percent of untreated strain A mice examined at 6 months of age are known to have lung tumor (*4*), in any study of pulmonary tumor induction in this strain concomitant observation of the lungs of animals of the same age is essential. Accordingly, at the end of each week of urethan treatment when six treated animals were autopsied, a similar number of control animals were examined also.

Grossly, no lung tumors were found in any of the control animals.

First, second, third, fourth, and fifth weeks. Several small foci consisting of two or three alveoli lined with prominent alveolar cells were present in some of the lungs. In each of three lungs, one mitotic figure was found in the alveolar cells. A fourth mitotic figure was present in bronchiolar epithelium.

Sixth, seventh, eighth, ninth, and tenth weeks. Several small foci consisting of two or three alveoli lined with prominent alveolar cells were present in some of the lungs. In each of three lungs one mitotic figure was found in the alveolar cells. Another mitotic figure was present in the bronchial epithelium of one lung.

Eleventh, twelfth, and thirteenth weeks. Every lung possessed 2 or 3 areas in which the alveoli were lined by cuboidal alveolar cells. In 2 animals, the involved areas consisted of from 15 to 20 alveoli and were believed to represent early adenomatous tumors.

BRONCHIAL AND BRONCHIOLAR INVOLVEMENT

The possible role of the bronchiolar epithelium in the histogenesis of these tumors was studied in two ways: 1) the proliferation and mitotic

activity in the bronchiolar epithelium and in the alveolar cells of urethan-treated animals were compared with those of the untreated animals, and 2) the topographical relationship of the fully developed tumor to a bronchiole and bronchus was ascertained.

No evidence of proliferative activity of bronchiolar epithelium was found in any of the experimental or control animals. Mitotic figures were observed in the bronchiolar epithelial cells of only nine animals, seven of these were in the lungs of urethan-treated animals and two in the controls. In four of the treated and one untreated animal, the mitotic activity was apparent in the epithelium of a bronchiole of a lung that was involved in a pneumonic process. In three urethan-treated animals and one control animal the mitotic figures were seen in the absence of an inflammatory process. Obviously the finding of only three mitotic figures on examination of hundreds of bronchioles from urethan-treated animals is of no particular significance. In contrast, the incidence of mitotic figures in the alveolar cells of urethan-treated animals was striking, since they were seen in every section of the lungs of animals treated for 3 weeks or more with urethan.

Three types of bronchial and bronchiolar tumor involvement were found in 18 animals that had received urethan for more than 8 weeks. In 12 animals 1 or more tumors were adjacent to a bronchus or a bronchiole, but there was no extension through the wall into the lumen. In the second group of 5 animals the respiratory bronchiole opened into an alveolar duct near a subpleurally situated tumor, but there was a distinct separation between the tumor and the termination of the bronchiole. In neither of these groups of tumors was there any direct or intimate relation or contact between the bronchial or bronchiolar epithelium and the tumor. In addition, 1 of the 5 animals of the second group showed the third type of bronchiolar tumor involvement. This situation was also found to obtain in another animal. In these latter 2 mice the tumor involved a group of alveoli and also was present in the lumen of an adjacent bronchiole (figs. 19–22). There are 2 possible interpretations to explain this finding: 1) that the tumor, having originated in the alveoli, secondarily extended into bronchiolar lumen, and 2) that the tumor originated from the epithelium of the bronchiolar tree forming a papillary projection into the bronchiole.

In deciding which one of the two possibilities is the more likely, four observations may be considered: 1) except for the presence of the tumor tissue within the lumen of the bronchioles, these two tumors were histologically identical to pulmonary tumors which did not involve any bronchioles; 2) on serial section study, the main body of the tumor in both instances was peripheral to the involved bronchiole; 3) many mitotic figures were present in the lining cells of alveoli surrounding the periphery of the tumor; 4) no atypism, anaplasia, or mitotic activity was seen in the epithelium of the involved bronchiole. These observations lend support to the concept that these two tumors originated from lining cells

of the alveoli and that the involvement of the bronchiole was simply a secondary extension of the tumor (figs. 13–22).

The low incidence of bronchial and bronchiolar involvement in this experiment is noteworthy. This is believed to be due to the fact that the treated animals were killed before sufficient time had elapsed for the tumors to extend into and involve the bronchioles and bronchi.

THE POSSIBLE RELATION OF INFLAMMATORY REACTION TO THE DEVELOPMENT OF THE URETHAN-INDUCED TUMORS

Since there was a possibility that pulmonary inflammatory reaction may have played a role in the development of these tumors, special attention was focused on the existence of pulmonic inflammation in both the treated and untreated animals. Not only was the presence of unmistakable pneumonic consolidation recorded, but preconsolidation stages of pneumonia and related inflammatory reactions such as peribronchial lymphoid infiltration, congestion, atelectasis, tracheobronchitis, granuloma formation, and fibrosis were also noted. The incidence of each of these in the experimental and control animals has already been described above but may be briefly summarized (table 1).

TABLE 1.—*Incidence of pulmonary inflammatory and allied conditions in normal and urethan-fed strain A mice as determined by weekly post-mortem examination*

Number of weeks of urethan administration	Experimental mice	Control mice
1	2	3
2	3	4
3	2	1
4	0	1
5	3	4
6	1	2
7	1	3
8	3	4
9	1	2
10	3	4
11	3	5
12	3	3
13	1	4
	26	40

Thus in approximately 50 percent of control and 30 percent of the urethan-treated animals, inflammation and related reactions of varying degree and type were manifest. Although the incidence of these reactions in the control animals was greater than in urethan-fed animals, the number of animals used in this experiment may not be sufficiently great to justify any significant conclusion in regard to the effect of urethan on the production or prevention of pneumonia in mice. It does show, however, that pulmonary inflammatory reaction may be of no more frequent occurrence in urethan-treated animals than it is in the untreated control animals. Furthermore, there appears to be no topographical relationship between the area of inflammatory reaction and the tumor. In 15 of 26 urethan-

treated animals that showed evidence of pneumonia, the inflammatory process and the tumor occurred side by side (fig. 23), whereas in 9 of these and the remaining 11 animals the tumor and the pneumonic process were histologically widely separated, even when they occurred in the same lobe (fig. 24).

Since the incidence of the inflammatory reactions described was less in the treated than in the untreated animals, it is believed that inflammation played no part in the development of these tumors.

Although atelectasis, included in the inflammatory processes listed, appeared to play no significant role in the development of the tumors, it may be argued that some preexisting atelectasis may have been obliterated by artificial expansion of the lungs during fixation by the intratracheal injection utilized in this experiment. However, in every case where atelectasis was identified grossly its presence was also apparent in the microscopic preparations. It is obvious that fixation did not interfere with the histologic demonstration of atelectasis in a large number of animals.

DISCUSSION

It seems clear from the material of this experiment that lung tumors induced in strain A mice by oral administration of urethan arise from alveolar cells. These cells become conspicuous after the first week of urethan feeding and by the third week manifest evidence of considerable mitotic activity, such a phenomenon being rarely encountered in the control animals. Under continued administration of urethan, groups of alveolar spaces become lined with alveolar cells that assume a neoplastic character, so that by the sixth week of urethan treatment typical adenomatous tumors may be found scattered throughout the lungs.

That these tumors arise from alveolar cells seems clearly indicated, not only by the observation of a remarkably great increase in mitotic figures and anaplasia of the alveolar cells of the lungs of treated animals as compared with the normal, but also by the complete absence of any such growth of bronchial and bronchiolar epithelium of the treated animals in which no increase in mitotic figures or anaplasia was found. Further, careful study of serial sections from the lungs in which papillary projections of the tumor were present in the lumina of bronchioles leads to the belief that even in these cases the tumor arose from peripheral alveoli and secondarily involved the bronchiolar tree.

It also has been shown that under the conditions of this experiment atelectasis and pneumonia played no significant role in the development of these tumors. The incidence of these inflammatory conditions in the tumor-bearing treated animals was less than it was in the non-tumor-bearing controls. While in some cases pulmonary inflammation and tumor occurred in the same microscopic fields, in other cases the tumor occurred independently of the inflammatory process.

That both the induced and spontaneous pulmonary tumors in mice arise in a similar fashion is generally accepted (*3*), but the histopathogenesis of these tumors has occasioned a great deal of controversy since Livingood

(*5*) first described spontaneous pulmonary tumors and advanced the opinion that they arise from the bronchus. Tyzzer (*6*), upon finding that the spontaneous tumors had no constant relationship to the bronchi, and that in one case a tumor was completely independent of a bronchus, presumed that they arose from alveolar cells. Jobling (*7*) traced the origin of a spontaneous pulmonary tumor to papillary outgrowths from the wall of a bronchiole. Slye, Holmes, and Wells (*8*) concluded that spontaneous tumors arose either from alveolar or bronchial epithelium, that the papillary tumors arose more frequently from alveolar epithelium, but that it was difficult to decide this point from the character of the tumor cells. Furth and Furth (*9*) in a large series of spontaneous and benzpyrene-induced pulmonary tumors found that they appeared to originate from alveolar epithelium. They were unable to observe a clear relationship between the tumors and the bronchial epithelium. Campbell's studies (*10*) on pulmonary tumors induced by tar-free dust and dust containing a small amount of tar led him to adopt the view that they usually originated in the alveolar cells as nonmalignant tumors and then changed more or less rapidly, depending on the degree of irritation, into a malignant type.

Magnus (*11*), in studying lung tumors produced in mice by the injection of 1, 2, 5, 6-dibenzanthracene into the lumen of the stomach, concluded that the nodules represented large intrabronchial papillomata which completely filled the lumina of dilated bronchi and that the stages in the development of the tumor could be traced from a small villous papilloma lying in a bronchus to a large nodule in which it was almost impossible to recognize the remains of the bronchial wall at the periphery. He believed that the tumors arose from the bronchial epithelium.

McDonald and Woodhouse (*12*) studied 71 primary tumors of mouse lung. A few of these occurred spontaneously while others were obtained from mice that had been subjected to various experimental procedures. They concluded that the lesions originated and spread by metaplasia and proliferation of the alveolar epithelium, bronchiolar epithelium also participating in the proliferative process.

Grady and Stewart (*13*) demonstrated that practically all the pulmonary tumors induced by subcutaneous injection of 20 methylcholanthrene and 1, 2, 5, 6-dibenzanthracene in strain A mice originated from alveolar cells and were not associated with an inflammatory reaction in the lungs. Orr (*2*, *3*), on the other hand, claimed that the tumors invariably arose in foci of atelectasis, chronic inflammation and fibrosis, and that the tumor cells originated from the bronchiolar epithelium.

Our observations confirm those of Grady and Stewart (*13*) in the same strain of mice and are at variance to those of Orr (*2*, *3*). We are unable to substantiate the latter's explanation of the origin of the tumor from bronchiolar cells that lined an alveolus, with subsequent loss of continuity between the terminal bronchiole and the tumor. In the lungs of animals treated with urethan, we have demonstrated mitotic activity in alveolar cells of patent alveoli. It is unlikely that differences in strain of animal could explain the different characteristics encountered, but it may be that

a preexisting or coexisting pneumonia in Orr's animals may have altered the picture. The discrepancy may well be due to confusion of the developmental phase of these tumors with a reaction that is occasionally seen in the lungs of mice having pneumonia. In some of these pneumonic animals, in addition to atelectasis and proliferation of the alveolar lining cells and exudation, we have seen a lesion identical to that described by Lorenz and Stewart (*14*). These investigators found in the involved area of the lung atypical acini lined by a single layer of cuboidal or columnar cells with basally situated hyperchromatic nuclei. They state, "the involved acini were usually separated from each other by a few pulmonary alveoli which were partially collapsed, showed fibrous thickening of their walls and contained a few macrophages. A bronchiole or large alveolar duct was frequently but not invariably associated with the apical, more deeply placed portion of this lesion. There was a striking resemblance between the epithelium of the acinous structures and that of the bronchi." Lorenz and Stewart were not able to determine whether these lesions were neoplastic or nonneoplastic in nature, but it was their impression "that the lesion may be bronchial dilatation and proliferation in association with inflammation rather than a neoplasm." Whatever its nature, this lesion can be easily distinguished both grossly and microscopically from lungs showing typical adenomatoid tumors.

In the majority of cases, the lungs possessing adenomatoid tumors are normal except for the presence of a few small tumor nodules.

We believe that much of the confusion could be avoided by differentiating between dilatation of bronchioles and proliferation of bronchial and bronchiolar epithelium as seen occasionally in pneumonic lungs, and by careful study of adequate numbers of tumor-bearing animals killed at varying intervals during the development phases of these tumors. Such an analysis, although time consuming, is indispensable to the proper understanding of the histopathogenesis of these tumors.

An interesting problem that has arisen during the course of this study is the nature of the cells that comprise the tumor. In hematoxylin and eosin preparations the tumor cells resemble epithelial cells. With the techniques used in this experiment, it could not be determined whether they are epithelial cells. Indeed, this problem involves the question of the existence and the nature of an alveolar-lining cell. The great increase in mitotic activity under urethan treatment of the parenchymal cells of the lungs which under normal conditions rarely show any mitosis, is significant, since it strongly suggests not only that urethan-induced tumors arise from alveolar cells but also that the alveolar cells do exist in strain A mice.

The induction of lung tumors in this strain of mice by a chemical agent that is employed in the chemotherapy of tumors in human patients merits further investigation.

SUMMARY

Oral administration of urethan to strain A mice produced pulmonary tumors microscopically identical with spontaneous tumors. The study

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of the histopathogenesis of urethan-induced tumors showed that they arise from alveolar structure and very probably alveolar cells, and not from bronchiolar epithelium, and that atelectasis and inflammation played no part in the development of such tumors.

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Plate 135

All photomicrographs are taken from sections of tissue fixed in Zenker acetic acid and stained with hematoxylin and eosin.

It should be noted that figs. 1–12 represent stages of the development of neoplastic foci as observed in different animals killed at weekly intervals. Figures 2, 6, 8, 9, 10, 11, and 12 are selected sections of serially sectioned paraffin blocks of lung, while figures 1, 3, 4, 5, and 7 are from lungs subjected only to single sectioning.

Figure 1.—Mouse F614, urethan, 1 week. Foci of increased cellularity (arrows) are present, but no atelectasis or inflammatory reaction. × 400.

Figure 2.—Mouse F751, serial section No. 228, urethan, 2 weeks. Note mitotic figure (arrow) in the alveolar cell immediately under the pleura. × 400.

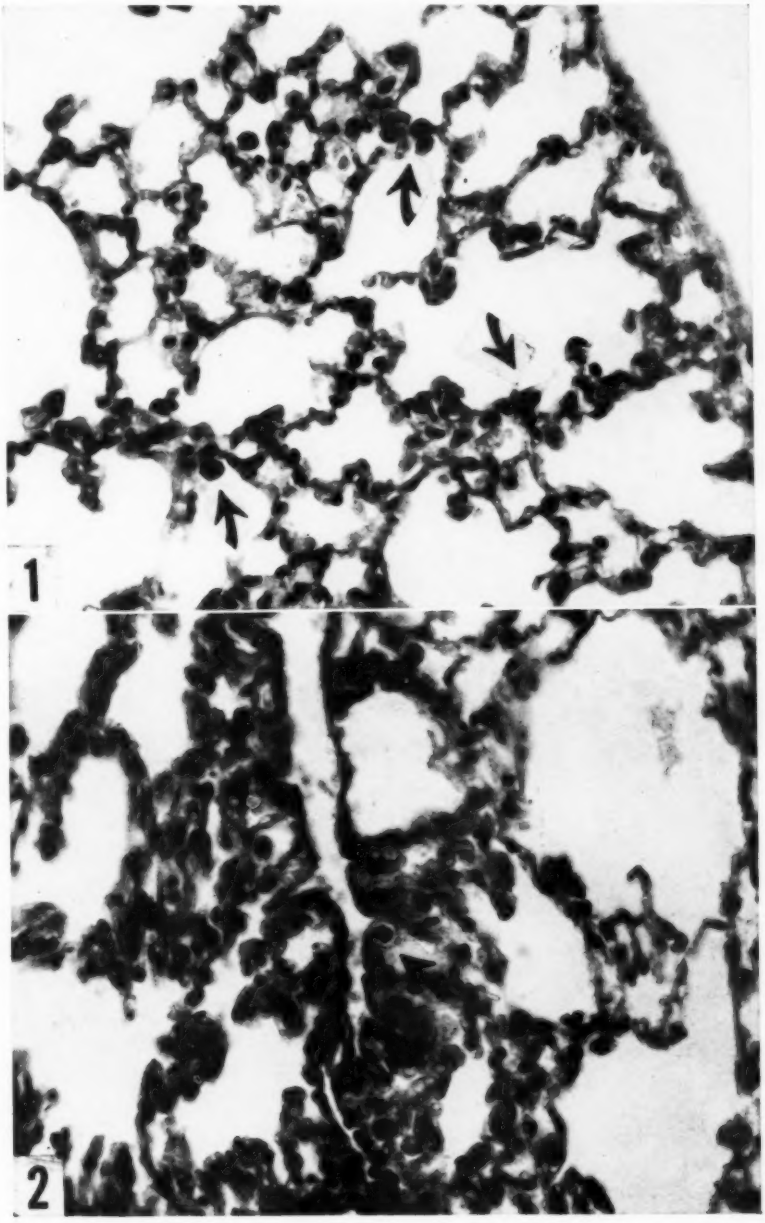

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PLATE 136

FIGURE 3.—Mouse F907, urethan, 3 weeks. In foci of increased cellularity (arrow) the cells involve two or three adjacent alveolar septa. × 400.

FIGURE 4.—Mouse F1173, urethan, 4 weeks. Four adjacent alveolar spaces are almost completely obliterated by newly proliferated cells (arrows). × 400.

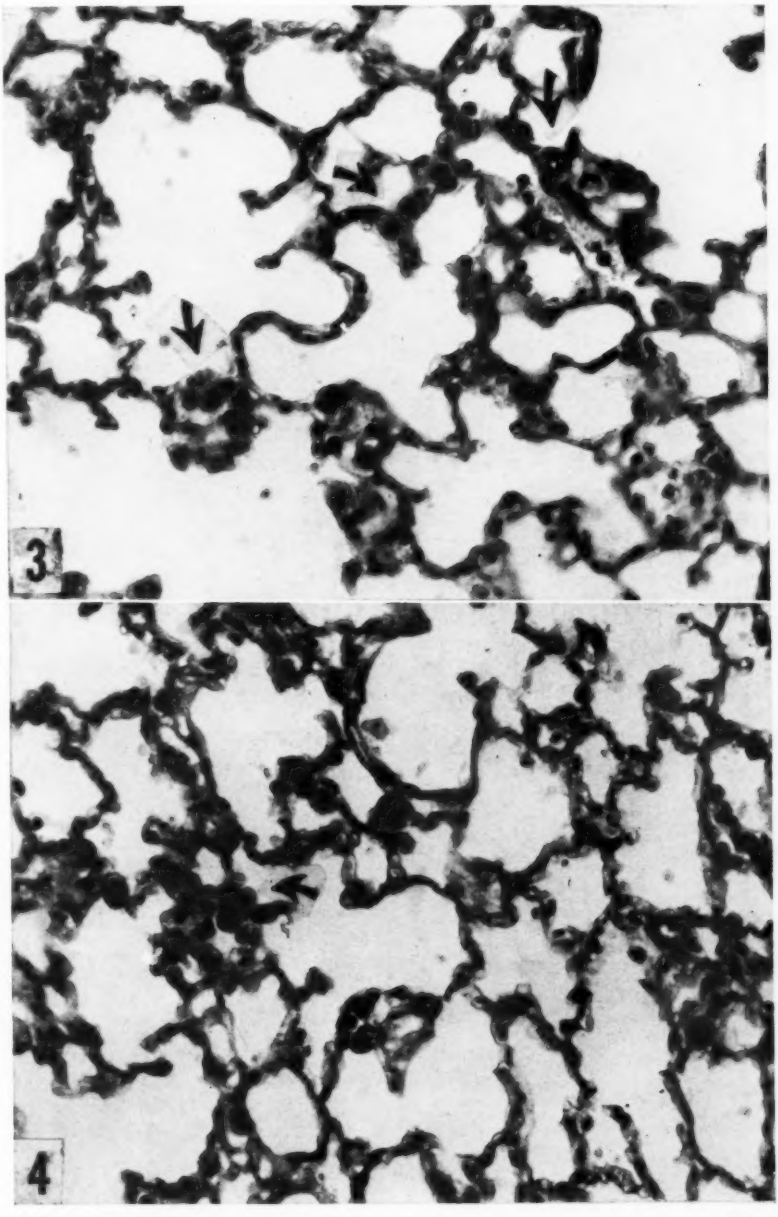
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PLATE 137

FIGURE 5.—Mouse F1482, urethan, 5 weeks. Focal areas of increased cellularity of alveolar wall. × 400.

FIGURE 6.—Mouse F1479, serial section No. 252, urethan, 5 weeks. A mitotic figure (arrow) is present in the alveolar wall. × 605.

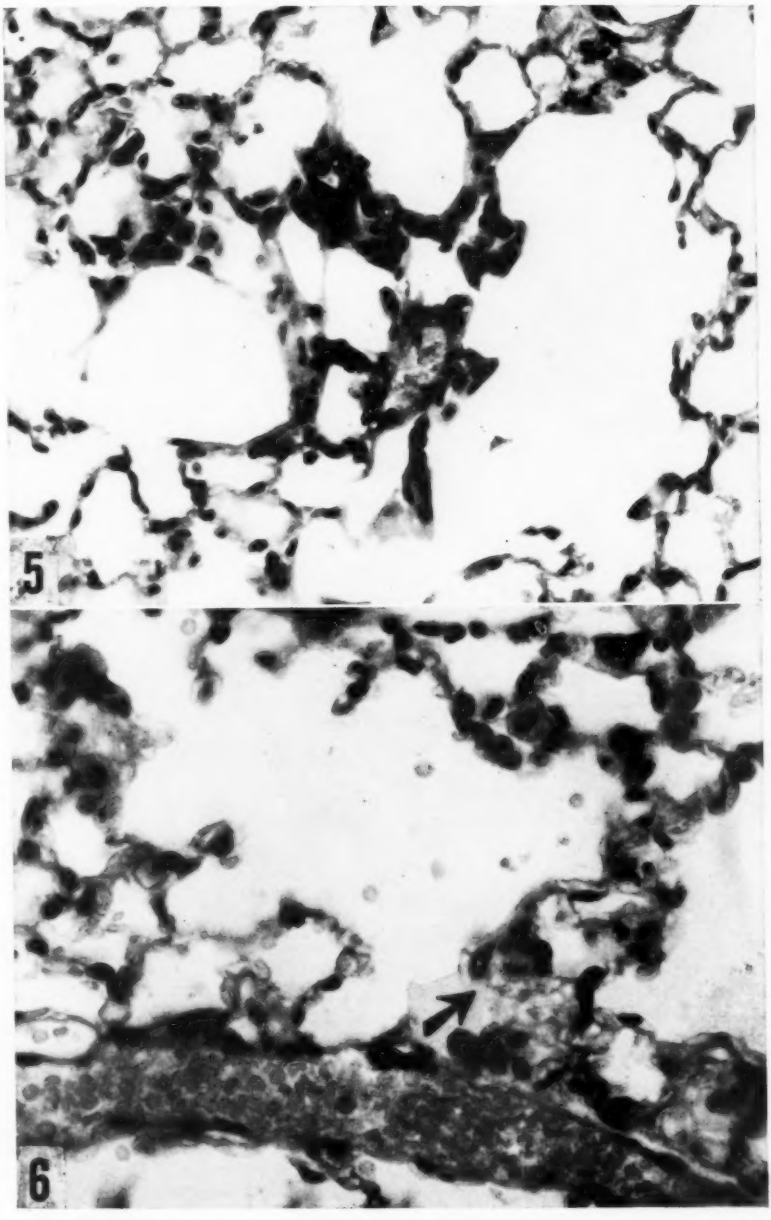
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PLATE 138

FIGURE 7.—Mouse F1687, urethan, 6 weeks. The earliest tumor recognizable grossly may be seen beneath the pleura. × 180.

FIGURE 8.—Mouse F1683, serial section No. 125, urethan, 6 weeks. Two patent alveolar ducts are seen (a. d.). Note the large number of alveolar cells forming a neoplastic focus. × 375.

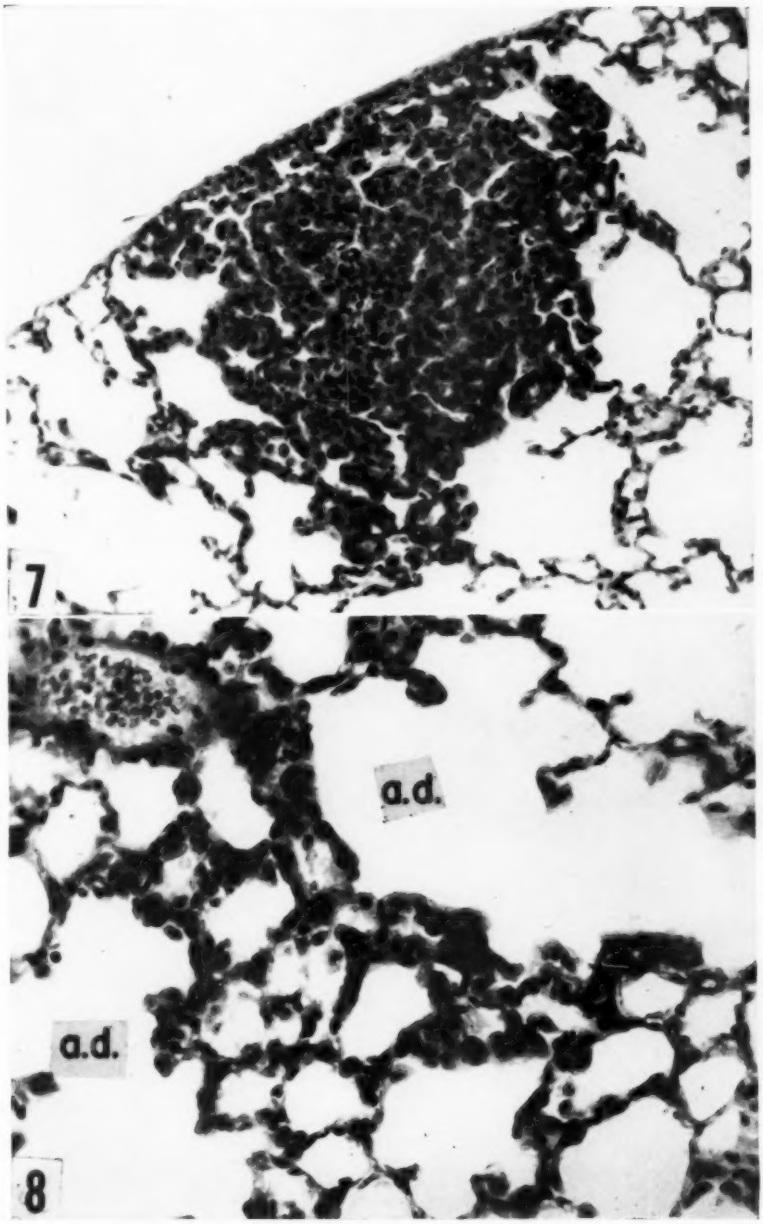
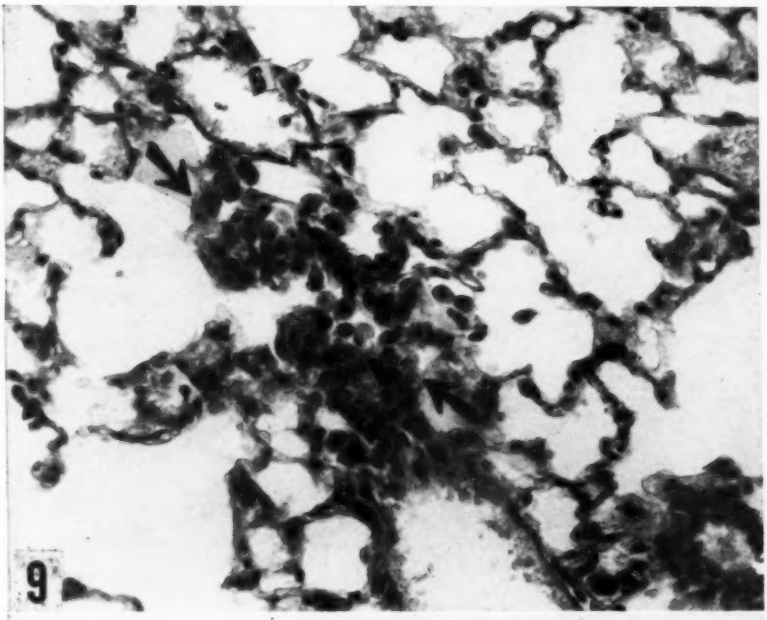

7
a.d.
a.d.
8

PLATE 139

FIGURE 9.—Mouse F1684, serial section No. 292, urethan, 6 weeks. Several adjacent alveoli show increase in alveolar cells. Two mitotic figures are seen (arrows). × 375.

FIGURE 10.—Mouse F2584, serial section No. 87, urethan, 8 weeks. Note the small neoplastic focus under the pleura. × 400.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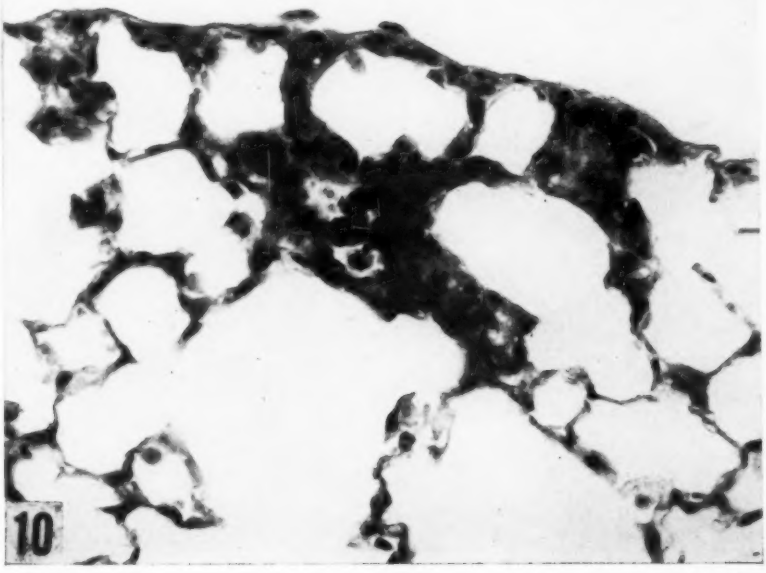
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PLATE 140

FIGURE 11.—Mouse F4305, serial section No. 160, urethan, 12 weeks. The peripheral portion of a lung tumor is seen. The alveolar spaces are retained but the septa are thickened. A mitotic figure (arrow) is seen in a cell protruding into an alveolar space. Note the similarity of this figure to figure 10. × 490.

FIGURE 12.—Mouse F4135, serial section No. 175, urethan, 12 weeks. In the center is seen an early neoplastic focus showing a large cell with a pale central area in its nucleus. A mitotic figure is seen in a cell that appears to be detached (arrow). × 500.

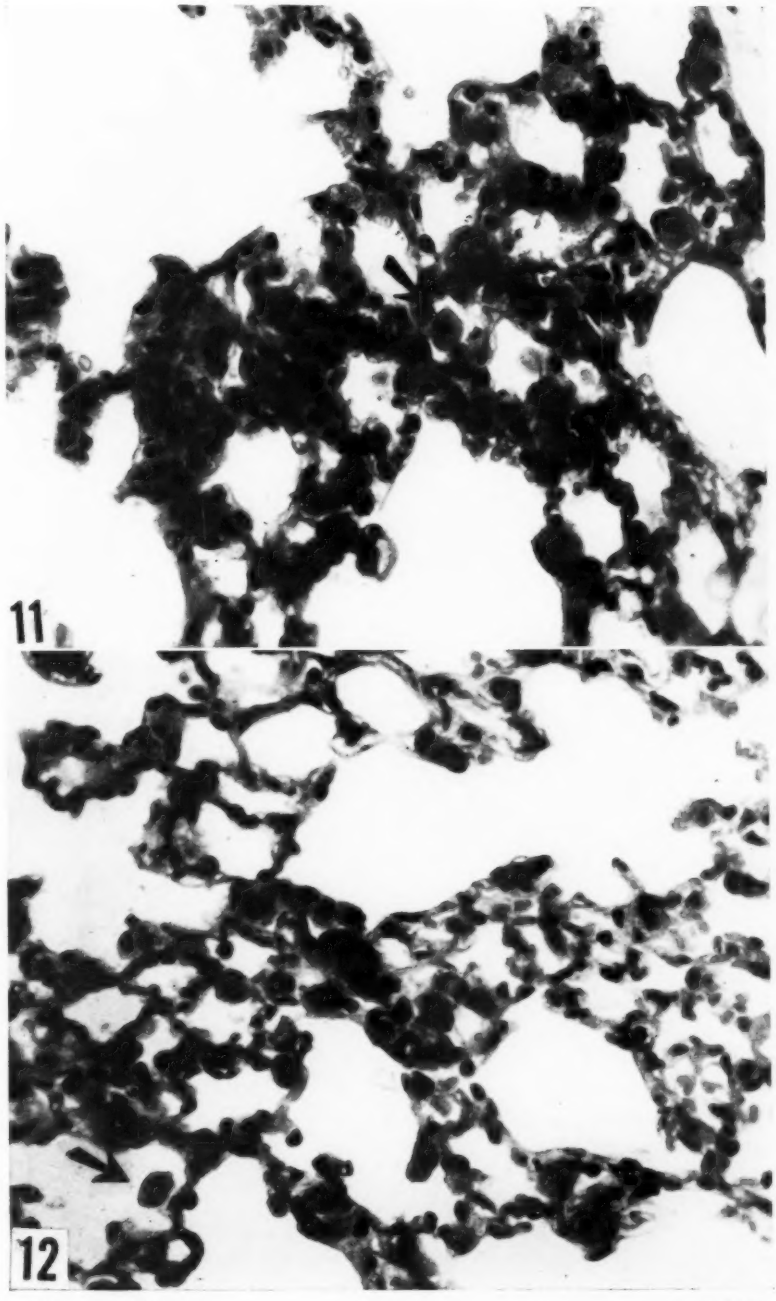
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PLATE 141

It should be emphasized that figures 13 through 22 represent selected serial sections taken from a single tumor of an animal that received urethan orally for 11 weeks.

FIGURE 13.—Mouse F3622, serial section No. 301, urethan, 11 weeks. Large alveolar cells line a few alveolar walls adjacent to a blood vessel (b. v.). Note the similarity to figure 4. × 350.

FIGURE 14.—Mouse F3622, serial section No. 303, 2 sections beyond figure 13, urethan, 11 weeks. The cell nest (arrow) is larger and has almost completely obliterated the alveolar space. The same blood vessel found in fig. 13 is seen (b. v.). Note the similarity of this figure to figure 5. × 350.

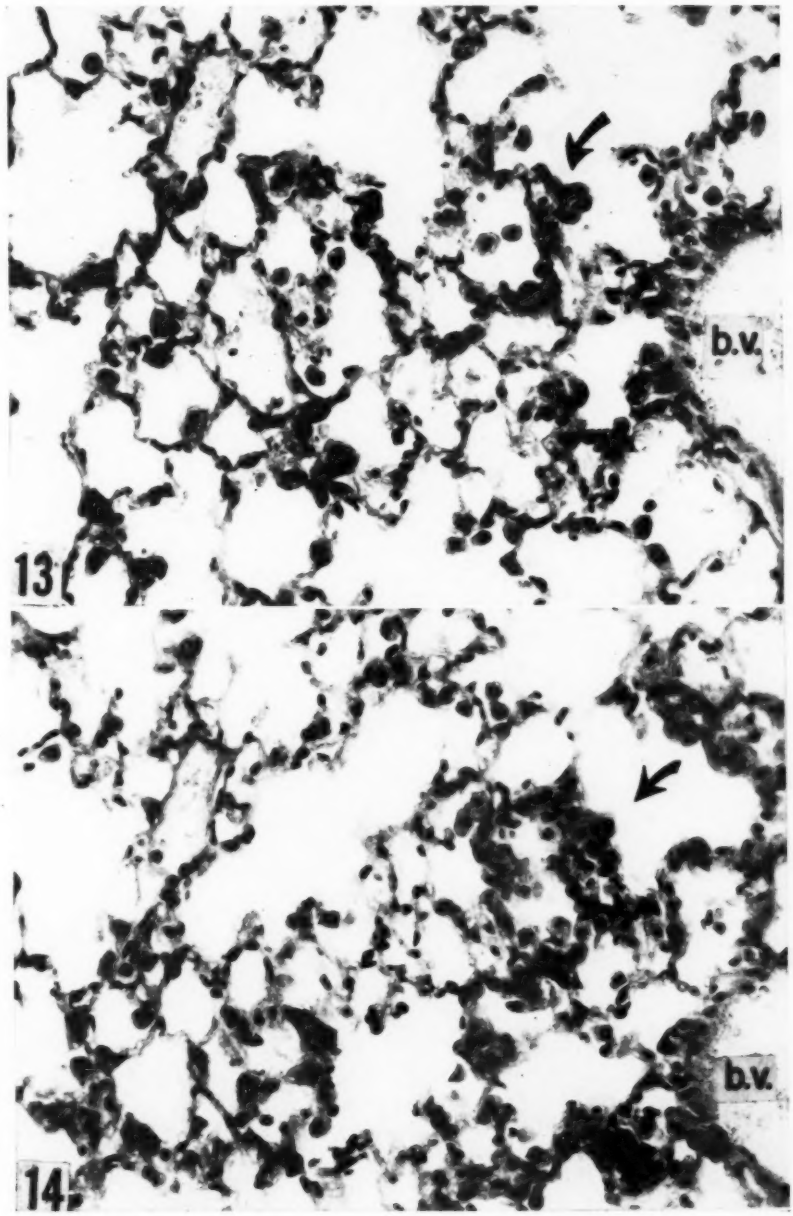
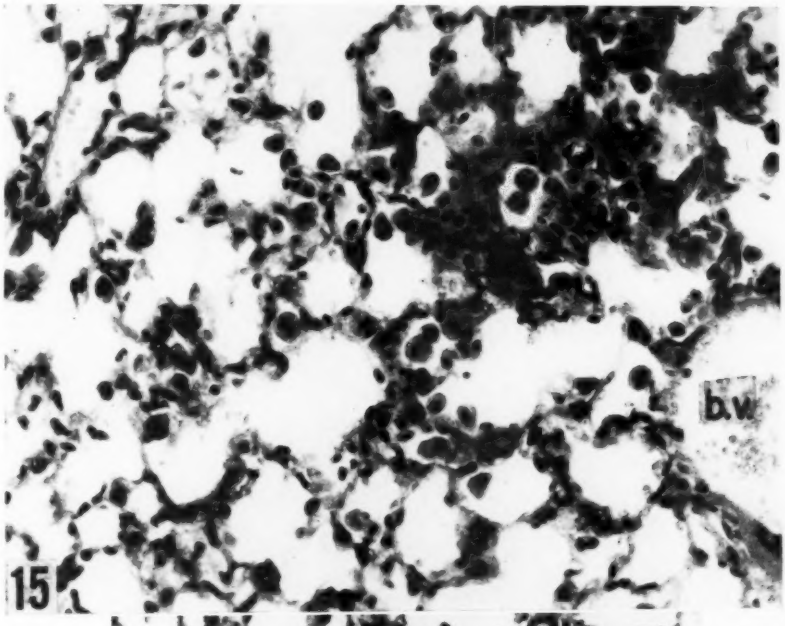

b.v.
13
b.v.
14

PLATE 142

FIGURE 15.—Mouse F3622, serial section No. 305, 2 sections beyond figure 14, urethan, 11 weeks. A neoplastic area may be seen near the same blood vessel (b. v.) which may be seen also in figure 14. Note the similarity of this figure to figure 9. × 350.

FIGURE 16.—Mouse F3622, serial section No. 309, 4 sections beyond figure 15, urethan, 11 weeks. The same neoplastic area is present. The tumor is quite distinct. A blood vessel (b. v.) is seen in the right lower corner which is the same as in figure 15. No bronchi or bronchioles are present anywhere near the tumor. × 205.


b.v.
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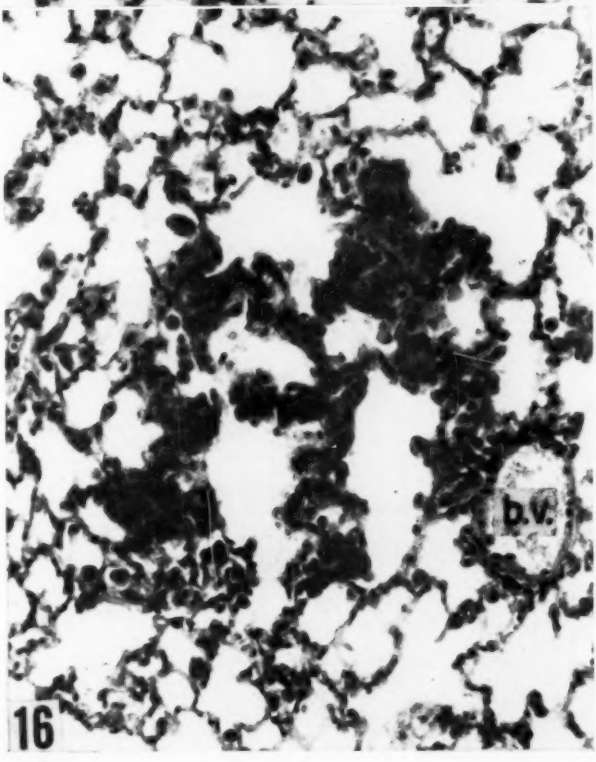
b.v.
16

PLATE 143

FIGURE 17.—Mouse F3622, serial section No. 311, 2 sections beyond figure 16, urethan, 11 weeks. The tumor occupies the alveolar spaces between alveolar ducts. A blood vessel (b.v.) may be seen in the right lower corner. × 350.

FIGURE 18.—Mouse F3622, serial section No. 323, 12 sections beyond figure 17, urethan, 11 weeks. The tumor is adjacent to a bronchiolar duct (b. d.). The same blood vessel (b. v.) is present as may be seen in figure 17. × 260.

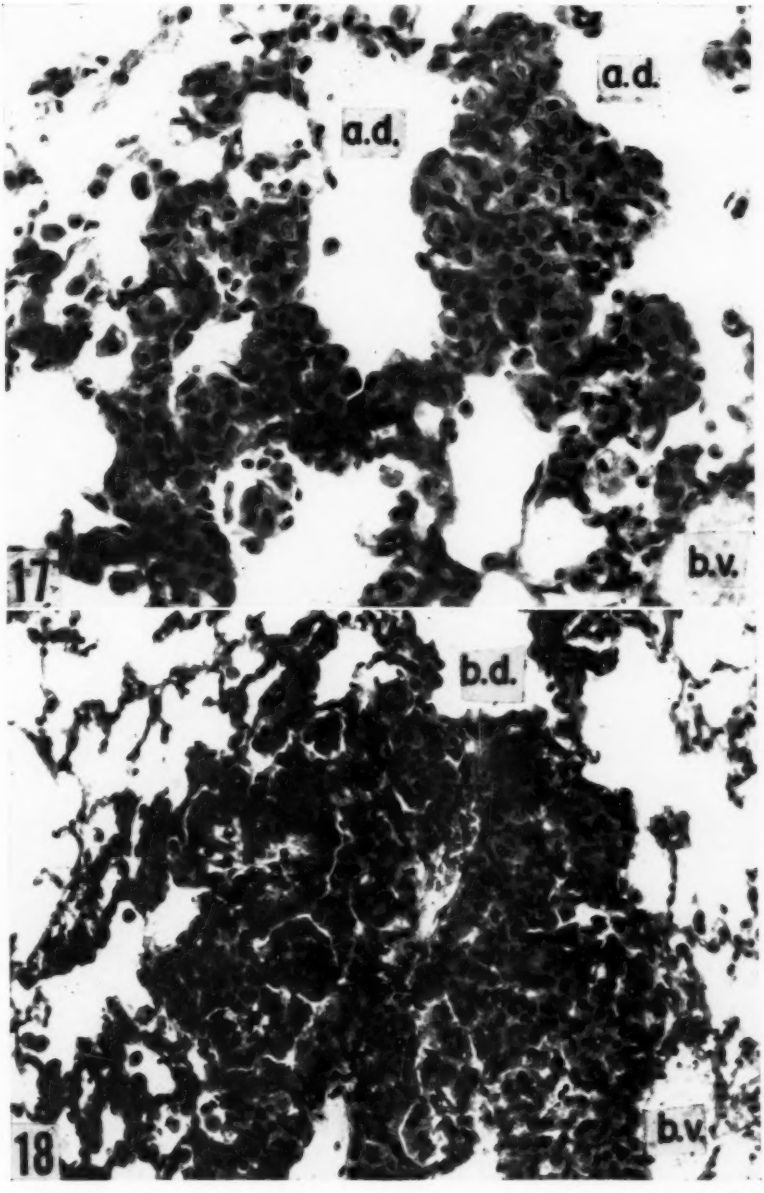

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PLATE 144

FIGURE 19.—Mouse F3622, serial section No. 327, 4 sections beyond figure 18, urethan, 11 weeks. The tumor is seen in the bronchiolar lumen (b. l.). × 205.

FIGURE 20.—Mouse F3622, serial section No. 332, 5 sections beyond figure 19, urethan, 11 weeks. The tumor occupies a bronchiole. Note the intact epithelial lining of bronchiole (arrows). × 205.

b.l
19

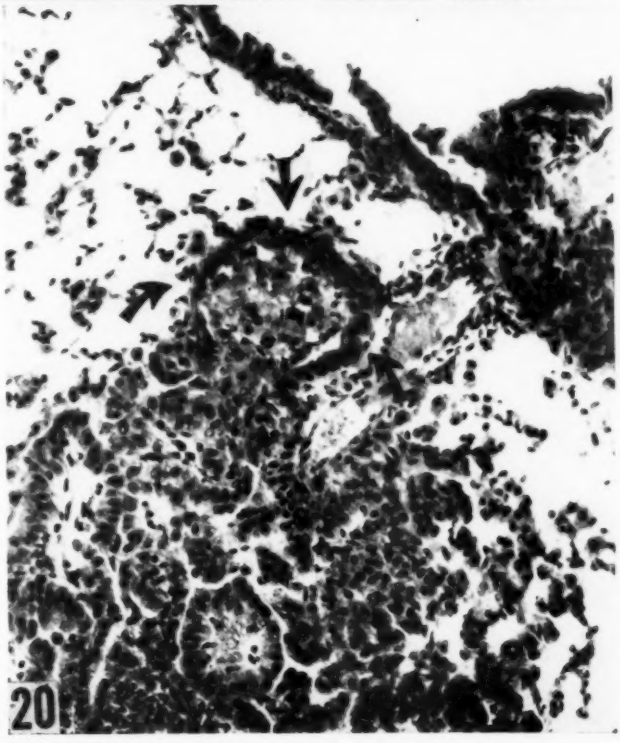
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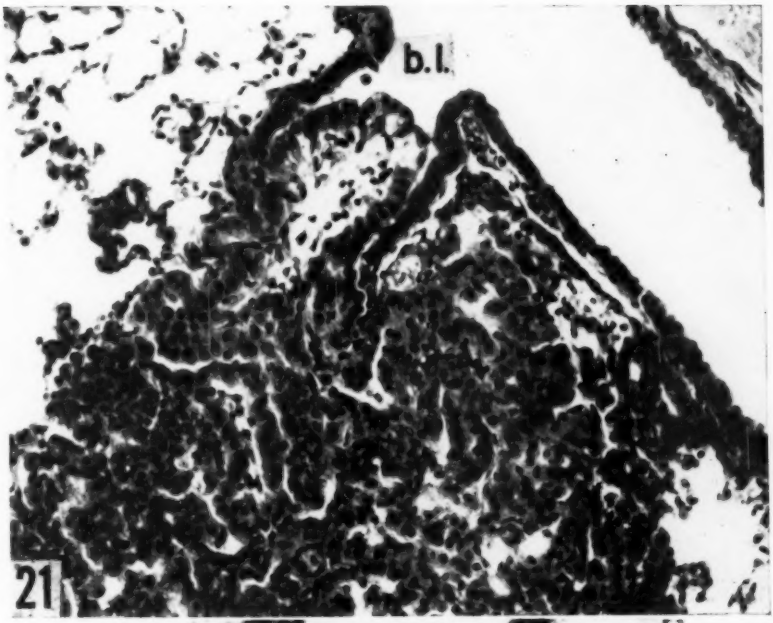
PLATE 145

FIGURE 21.—Mouse F3622, serial section No. 339, 7 sections beyond figure 20, urethan, 11 weeks. Another view of the tumor within the bronchiolar lumen. × 205.

FIGURE 22.—Mouse F3622, serial section No. 351, 12 sections beyond figure 21, urethan, 11 weeks. The main body of the tumor is peripheral to the bronchiole seen in figure 21 and is adjacent to another alveolar duct (a. d.). × 205.


b.l.
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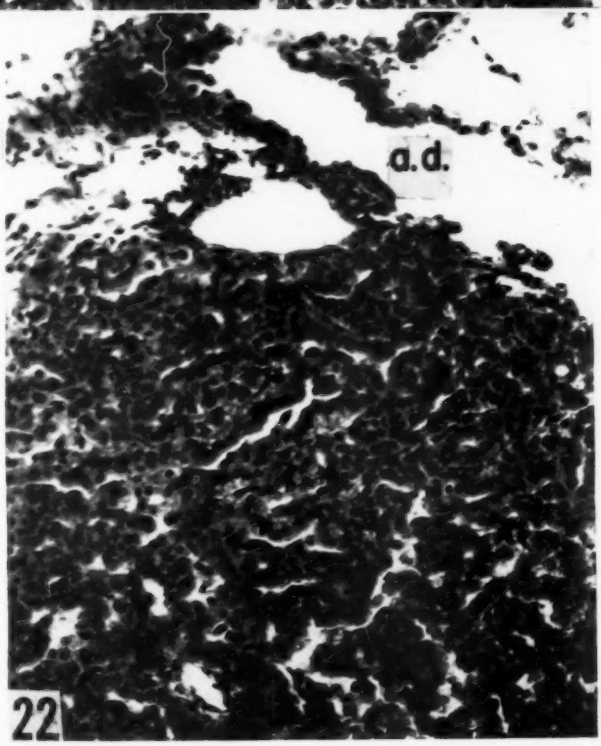
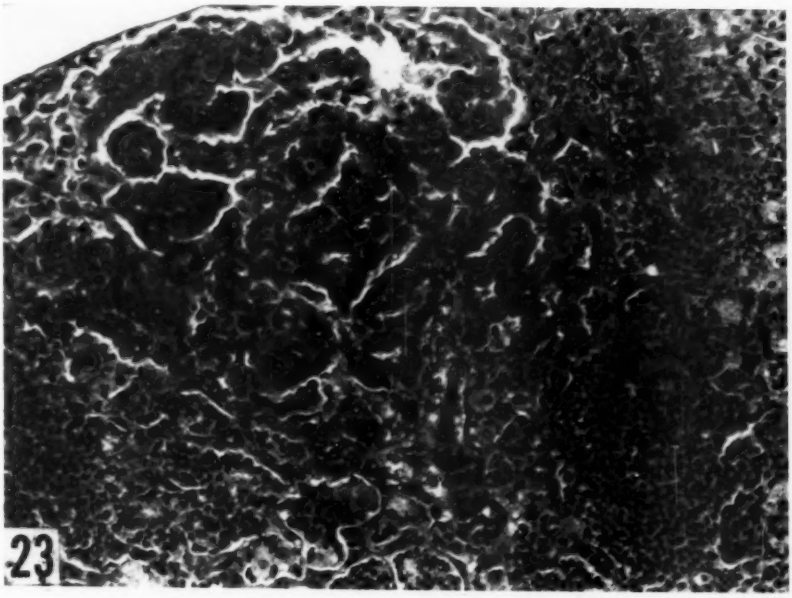

a.d.
22

PLATE 146

FIGURE 23.—Mouse F4136, urethan, 12 weeks. A tumor associated with pneumonic focus. × 205.

FIGURE 24.—Mouse F4136, urethan, 12 weeks. From another lobe of the same lung shown in figure 23. The tumor is not associated with any pneumonic process.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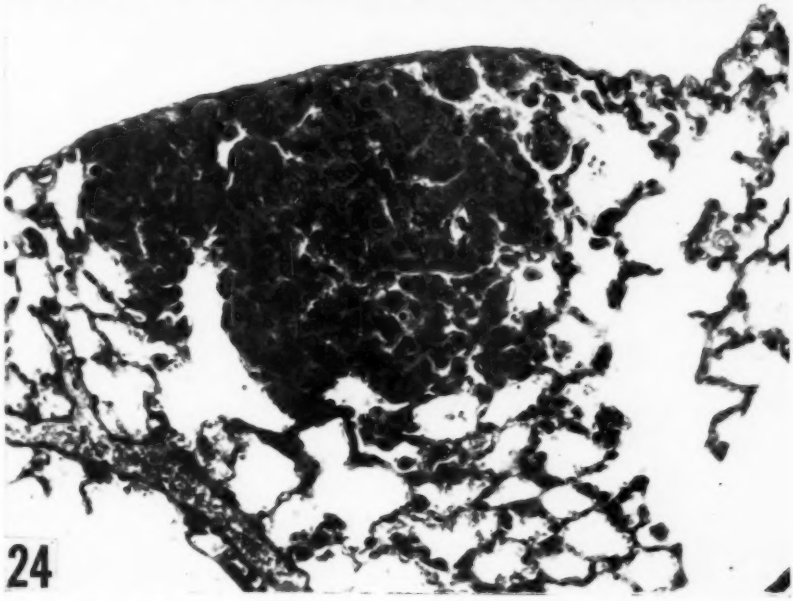
24

OBSERVATIONS ON THE CULTIVATION OF SURGICAL SPECIMENS OF HUMAN OVARIES *IN VITRO* [1] [2]

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INTRODUCTION

There is a growing tendency among tissue culture workers to believe that *in vitro* observations on living human tumors may prove increasingly valuable in determining the diagnosis, cellular origin, and virulence of neoplastic tissues. A study by Murray and Stout (*1*) on sympathicoblastomas provides an excellent illustration of the emerging importance of these views, since tissue cultures were shown by these authors to reveal highly diagnostic patterns within 24 to 48 hours. The scope of opportunity for pathologic studies has been re-emphasized by Sano and Gault (*2*).

Tissue culture procedure to be employed for studies on neoplastic tissue should be determined by the objectives in view. Thus, investigations designed to explore the possible merits of carcinostatic drugs, the effect of ionizing radiations and similar problems may require the development of a pure cell strain. The argument in favor of this approach has recently been re-enunciated by Shelton (*3*). A strong defense, however, can be made by workers whose primary concern is the diagnostic problem involved in the interpretation of tumors of borderline type for an intensive study of the initial outgrowth from explants of surgical specimens maintained in a simple medium. It was with this object in mind that data were accumulated in a somewhat routine fashion on an unselected series of human ovaries collected as surgical specimens. Obviously, the materials described in this preliminary report might have yielded a far richer fount of information had medium replenishment, variations in the composition of the medium, and subculture procedures been introduced. Nevertheless, the information presented with the use of a simple culture procedure, not even involving the renewal of the fluid nutrient medium during a period of observation of 2 to 4 weeks, may serve particularly well to illustrate the potentialities of the method for experimentally-minded pathologists. The galaxy of cells that emigrate from explants of surgical specimens, their relative numbers, rates of migration, differentiation and regrouping in the outgrowth, together with the staining reaction, proliferative and synthetic capacity of the nuclei, may provide a rich source of new data for the study of neoplasms.

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MATERIALS AND METHODS

Specimens from the operating room were delivered to the tissue culture laboratory usually within less than 1 hour after operation. While no systematic study has been made of its antiseptic merit, it was considered useful to place the tissues in Gey's solution containing Penicillin G 2,000 units per ml. until fragments were cut for explantation.

Three explants measuring approximately 1 mm. square were incorporated in a clot consisting of equal parts of heparinized cockerel plasma and embryonic juice derived from chicks incubated for 8 days on a No. 1 cover glass measuring 12 by 50 mm. As soon as the clot had firmly set, the slides were introduced into sterile Pyrex test tubes measuring 15 by 150 mm.; the tubes were closed with cotton plugs and left undisturbed for 3 hours. Three milliliters of a nutrient consisting of pleural or ascitic fluid (50 percent), Gey's solution (45 percent), embryonic extract (5 percent), and penicillin 1,000 units per ml., were then introduced and the tubes closed with No. 0 rubber stoppers. Cultures were then placed in a drum rotating at 8 revolutions per hour.

Experience in this laboratory has shown that ascitic and pleural fluids of human origin provide an excellent medium for cultivation of human cells. It is not yet known whether the merits of these materials depend primarily upon the presence of homologous proteins, but evidence has been found for the fact that ascitic fluid derived from patients with a diagnosis of *carcinomatosis peritonei* is somewhat superior to human umbilical-cord serum and very much more useful than ascitic fluid of nonmalignant origin in the cultivation of human adult epidermis [Ulloa-Gregori, Blocker, Nowinski, and Pomerat (*4*)].

The body fluids used in preparing the nutrient media employed in the cultivation of the nonneoplastic ovaries are given in table 1. Cultures of the ovarian tumors were treated with pleural fluid W (acute, aseptic inflammatory exudate) and ascitic fluid M (congestive heart failure). These fluids were proved adequate to support a luxuriant growth of the autologous cells. The presence of 3 ml. of fluid phase medium not only may be adequate to supply nutrients and for the reception of catabolic products but may assure a system in which the environment need not be disturbed for a 3-week study period.

Cultures were observed daily. Special importance was attached to the first evidence of emigration of the cells, particularly those of epithelial type.

Photographs were made of living cultures with the use of a phase system (American Optical Co.), including 8 mm. and 1.9 mm. dark phase objectives. Cinematographic records, with the use of a time-lapse device (Rolab Co.), were made at intervals of 4 and 8 frames per minute.

Preparations were fixed at times when significant events were observed under living conditions in the history of the culture. The fixative consisted of methyl alcohol (5 min.), followed by the stain proposed by Jacobson [*cf.* Orr, Hardy, and Pomerat (*5*)], which consists of the May-Grünwald mixture followed by Giemsa. This staining method may

TABLE 1.—*Data regarding nonneoplastic ovarian tissue cultivated* in vitro

Patient	JSH number	Age	Race	Diagnosis	Number of explants cultured	Body fluid used as nutrient[1]
N. W.	7314–F	27	Caucasian	Chronic cervicitis, corpus interim cyst, normal ovary.	150	St
L. C.	6767–G	40	Colored	Carcinoma of Bartholin's glands, normal ovary.	240	A
M. E.	9199–G	30	Caucasian	Chronic salpingitis, chronic cervicitis, follicular cysts of ovaries.	60	T
A. C.	11727	38	Colored	Myoma uteri, hydrosalpinx (left) normal ovary.	150	T
W. J.	11735–G	40	do	Chronic salpingitis, fibromyoma uteri, chronic cervicitis, normal ovary.	150	T
Total					750 explants in 250 roller tubes	

[1] St=*Ascitic fluid*—Adenocarcinoma of the ovary. A=*Pleural fluid*—Essential hypertension. T=*Ascitic fluid*—Carcinoma of the head of the pancreas.

prove useful in locating areas in cells containing ribonucleoprotein *versus* desoxyribonucleoprotein.

NONNEOPLASTIC OVARIES

In the course of studying surgical specimens, 5 nonneoplastic ovaries were cultivated *in vitro*. The significant data regarding these specimens are summarized in table 1. Typical epithelial outgrowth in compact sheets was never seen to develop from the specimens listed in this series. Large isolated epithelioid cells, however, were regularly found. While no attempt was made to segregate follicles in the process of development or special effort made to disclose the presence of viable epithelium, it would seem reasonable to suppose that in the course of culturing more than 750 explants, totaling some 750 sq. mm. of ovarian tissue, typical epithelial sheets might have been obtained in these experiments. While epithelium from the normal adult ovary has been successfully cultivated [Gaillard (*6*)], it appears that under the conditions of our experiments the proliferation of epithelium was not favored. If our medium or technique of management was not particularly useful for the establishment of nonneoplastic epithelium, the proliferation of spindle elements was certainly assured. In practically all of the 250 roller-tube cultures of explants from the tissues of the 5 subjects in this part of our series, rich outgrowth of fibroblast-like cells was found. The use of body fluids in the nutrient medium from both malignant and nonmalignant sources, as indicated in table 1, did not alter the direction of differentiation. Characteristic results are illustrated in figures 1–6.

Current work in progress is designed to isolate follicles from the ovaries

of young women ovariectomized for pathologic processes, not primarily involving the gonads, in order to define the growth-stimulating properties of our medium and the validity of our procedure in handling such material.

Benign Tumor of the Ovary

Case A. M.—JSH No. 4450–C, 46-year-old colored woman, revealed the presence of a Brenner tumor of the left ovary.

The tumor was composed of two constituents, namely, nests of epithelial cells resting in a fibrous connective tissue stroma (fig. 7). The nests of epithelial cells were characterized by fairly large nucleoli surrounded by a moderate amount of acidophilic cytoplasm (fig. 8). The cytoplasm showed a very slight vacuolization in an occasional cell. The nuclei were characteristically vesicular, many having a small but prominent nucleolus. The nests were sharply delineated by fibrous connective tissue which was characterized by elongated spindle cells with long, somewhat hyperchromatic nuclei. Within the cell nests no mitoses were observed.

Tissue from the surgery was washed for 4 hours in Gey's solution containing 2,000 units per ml. of Penicillin G. Selected portions believed to represent actively growing tumor tissue were cut into 1-mm. square fragments, and 300 explants were set up in plasma clots in 100 roller-slide cultures. After 2 hours, 3 ml. of nutrient fluid containing 50 percent pleural fluid (W) were added to each tube. This was never replenished during the entire period of the study (27 days). Roller tubes were allowed to lie in a horizontal position for 48 hours at 37° C. in order to set the plasma clot, after which they were rotated at 8 revolutions per hour in an incubator at 37° C.

Examination of living cultures showed no outgrowth on the 2d day, but by the 4th day cellular migration could be seen in most preparations. This consisted primarily of spindle cells of the fibroblastic type. Mitotic activity was frequent at this time and persisted throughout the period of study (27 days). Photographic records made from a preparation fixed on the 6th day showed two characteristic types of cellular aggregation: (*a*) Loosely organized spindle cells with frequent mitotic figures (figs. 9 and 10); (*b*) An epithelial sheet (fig. 11) composed of cells with densely staining nuclei showing moderate variations in size and the presence of usually one dense nucleolus. The cytoplasm was lightly stained and showed numerous small, clear, circular and oval areas. In contrast to the spindle cells, mitoses were never observed in the epithelial sheets of stained preparations.

Observations on stained preparations fixed after the 8th day of incubation did not reveal epithelial outgrowth. Such cultures, however, consisted of a luxuriant outgrowth of spindle cells which, in areas close to the explants, consisted of dense, narrow, interlacing patterns, while at the periphery the corresponding elements appeared broader and formed sheets that might be characterized as constituting a "pseudo-epithelium." The nuclei of these cells showed considerable variations in size and form. Nucleoli were variable but generally consisted of from three to five small,

dense spherical masses. Mitoses were exceedingly common. Intranuclear vacuoles were relatively frequent. The cytoplasm was moderately basophilic and showed a few inconspicuous acidophilic granulations. These features are represented in figures 12 and 13.

It must be recalled that the fluid nutrient medium was never replenished in this series. By the 27th day, some cultures resembled those fixed on the 8th day although they appeared to have greater variations in the nuclear size of the spindle cells, and evidence of cellular disintegration was fairly common. The presence of relatively healthy cultures on the same roller slide as those bearing outgrowth showing almost total cellular disorganization remains unexplained, but it appears likely that these differences may have been due to the potentiality of the initial explant or to physical injury to the specimen in handling.

In comparing the histologic sections, which were prepared by the paraffin method and stained with hematoxylin and phloxine stain, with the material grown in tissue culture, one is impressed by the striking similarity of the two. The nests of epithelial cells that grew in tissue culture had vesicular nuclei, some with one and some with two nucleoli. The cytoplasm was finely granular and in some places vacuolated in the same way as in the fixed-tissue section. The nests of epithelial cells were sharply delineated from the surrounding connective-tissue stroma. The stroma itself, in tissue culture, was only slightly different from that observed in the section. In cultures, the stromal cells were less closely packed, the nuclei tended to become more rounded and the cytoplasm of the individual cells more abundant and more easily distinguishable than in the paraffin sections. Mitotic figures were observed in the tissue culture; this feature was absent in the connective tissue observed in paraffin sections.

One can best summarize the observations of the tumor which was studied by the two methods—by fixed section and with tissue culture—by stating that the similarity of the tumor in both instances was striking. As a matter of fact, differentiation of the tissue culture of the tumor from the one studied in the histologic section is quite impossible if one approaches the problem objectively. The only minor difference noted was the presence of mitoses in the fibrous stroma that was grown in tissue culture, a feature commonly encountered in connective-tissue cultures.

Malignant Ovarian Tumors

1. Tissue from patient A. R.—JSH No. 4945–G, age 47, a colored woman, with a diagnosis of *papillary cystadenocarcinoma* of the ovary, was utilized for a study of the natural history of cellular transformations *in vitro* with and without the addition of hormones in the fluid nutrient.

Studies of sections of the tumor showed considerable ovarian stroma and some fibrous tissue with focal areas of necrosis. The walls of the cystlike spaces observed in the gross specimen were lined, for the most part, with a cuboidal cell but in some areas the epithelium assumed a columnar pattern. In a few areas, there were isolated strands of connec-

tive tissue surrounded by cuboidal epithelial cells. This was interpreted as being the papillary projection into the cystic cavity (fig. 14). A more detailed study of the cell type of the epithelium (fig. 15) lining the neoplasm revealed, in general, hyperchromatic nuclei. However, the presence of mitotic figures was not a conspicuous feature. There was some degree of variation in the shape of the individual nuclei. The cells showed a moderate amount of cytoplasm. In some areas the cytoplasm tended to be vacuolated and in other areas was more homogeneous in nature.

The plan of the tissue culture study involved:

Roller slides each with 3 pieces of ovarian tissue	Fluid nutrient containing 50 percent (W) pleural fluid 3 ml. per roller tube
45	No hormone.
20	Na testosterone SO_4—100 gamma per ml. (Ayerst, McKenna & Harrison).
20	Na estrone SO_4—12.5 gamma per ml. (Ayerst, McKenna & Harrison).

Tissue explants were allowed to stand in a plasma clot for 2 hours before the addition of fluid nutrient. Rotation of the tubes was begun after they had remained undisturbed in the horizontal position for 48 hours. The fluid medium was not changed during the course of the 21 days of the study.

A very scant outgrowth of spindle cells was seen to have appeared before 48 hours of incubation. On the fifth day, one slide from the control series showed an epithelial sheet. This was employed for still photomicrographs and time-lapse cinematographic records, using a phase contrast system. Figure 16 shows a living epithelial sheet formed of large polyhedral cells. The cytoplasm of many elements showed vacuoles indicative of pinocytosis (p) associated with an undulating cell border (b); mitoses (m) were common within these sheets. Figures 17–20 are valuable for the demonstration of the form of nuclei and their conspicuously large nucleoli. The binucleate cell in figure 18 shows the nuclear membrane in sharp outline in the living unstained preparation. Perinuclear aggregations of mitochondria and dense spherical granules were typical of the epithelial elements.

After 14 days of incubation, cultures not containing hormonal additions were characterized by the presence of loosely organized oval or polyhedral cells of enormous size (figs. 21 and 22). Some of these elements measured over 250 μ by nearly 200 μ; the nucleus of some being approximately 60 by 40 μ. Nuclei were usually central, and cells occasionally were binucleate. The nuclear area was surrounded by coarse spherical granules. The cytoplasm, under low magnification, was marked by criss-crossed striae, which were probably due to the presence of tonofibrillae. The periphery of the cells was marked by irregular undulating membranes suggestive of high mobility.

Epithelial outgrowth was found in only 3 tubes out of a total of 45 control cultures and in 1 culture containing estrone out of a total of 40 roller tubes

treated with hormone preparations. Since these particular cultures were utilized for photographic records, they were eliminated from the series. By the 21st day of the experiment, all remaining cultures were fixed. These all had shown vigorous outgrowth of spindle cells. Migration of these elements was conspicuous during the entire course of the study but mitotic activity became markedly reduced after the 12th day. Variations in nuclear size and staining reaction on the 21st day may have reflected some nutritional injury due to the fact that the fluid medium had not been replenished.

Cultures containing hormonal preparation were not found to show any features conspicuously different from those of the control series, with the exception of one slide grown in medium containing the estrone compound, in which there was an extraordinarily extensive epithelial outgrowth. The culture measured 12 mm. across the longest axis on the 11th day of incubation (fig. 17), the explant being approximately 3 mm. wide along this line. A composite picture (figs. 24–27) of cells in the outgrowth of this culture shows the compact arrangement of the epithelial sheet, the characteristic nuclei, the giant and multiple nucleoli, which were relatively common, and the various stages of mitotic activity. At higher magnification, records were made of the position and outline of nuclei (figs. 28–33). In the area of closely packed cells, there was considerable variation in nuclear size (figs. 29 and 30), outline (fig. 28), and content. The arrangement of filamentous mitochondria is especially well seen in figures 28, 31, and 33. These photographs also illustrate the characteristic distribution of highly refractile, spherical granules, probably representing fat droplets. The marginal outline and contacts between adjacent cells are shown in figures 21, 32, and 33. We believe that the assemblage of the characteristics represented in these photographs constitutes the pattern of the cell species involved in the production of this tumor.

Photographs of the preparation described in the preceding paragraph but stained with Jacobson's method show the arrangement of cells in a compact zone (fig. 34), a looser arrangement near the periphery (fig. 35), and the pattern of cells at the margin (fig. 36). Variations in size (fig. 34) and in the form of the nuclei (fig. 37) as well as the dense nucleolar content can be seen.

Cultures of the control series were employed for making cinematographic records. A time-lapse device made possible records of activities for projection at a speed acceleration of 120 times. The process of cell division in components of the epithelial sheet of a 5-day culture was recorded together with the behavior of the organelles in a binucleate cell.

Using a perfusion system described by Pomerat (7), the activities and reactions to hormones of a typical cell of giant size from a 13-day culture shown in figure 21 were followed. Motion picture records were made for 1 hour during gentle perfusion with a *control* fluid nutrient prepared to contain 45 percent Gey's solution. The perfusate was then changed to a similar nutrient fluid but containing 200 gamma per ml. of sodium testosterone sulfate (Ayerst, McKenna & Harrison).

Records made during a 3-hour period did not indicate that the hormone affected the cytoplasmic movements at the border of the cell or the behavior of the organelles. In an attempt to remove the hormone-containing solution, the chamber was perfused for 22 hours with the control nutrient fluid. Records of cellular activity were made for 3 hours at the beginning and also at the end of this period of perfusion. Finally, a solution containing 40 gamma per ml. of sodium estrone sulfate (Ayerst, McKenna & Harrison) in the nutrient fluid was perfused through the chamber for a 3-hour photographic recording period. Immediately upon the introduction of the estrone, the mobility of the cell appeared to be considerably increased. Large leaflike processes on the expanded surface were seen to spread out against the glass contact. These activities persisted throughout the recording period.

While inconclusive in themselves, these results have proved the feasibility of making direct observations on the action of chemicals upon living malignant cells under highly controllable conditions. The effect of sterol compounds at various concentrations on the activity of the mobile components of individual cells can be recorded with precision. Such work, however, will require the establishment of reproducible base lines, including data on the effect of variations in the pH and the osmotic pressure of the medium as well as the action of agents influencing surface tension. Hormones to be tested should include molecules of well-established endocrine activity along with controls consisting of biologically inactive sterols.

In comparison with the histologic specimen, the study of the tumor grown in tissue culture showed sheets of epithelial cells that revealed numerous mitotic figures and much smaller nuclei. The nucleo-cytoplasmic ratio was much greater. The presence of mitoses was a conspicuous feature, and one gets the impression that these tumors, once they get into the open on tissue culture media, proliferate very extensively. A study of some of the stromal cells grown from this tumor showed no conspicuous changes from those that one would have expected. The cytoplasm was elongated and spindle-shaped. The nucleus located near the center of the cell was ovoid and somewhat hyperchromatic, with the chromatin assuming a somewhat mottled form and with nucleoli being present in some of the nuclei in tissue culture. The presence of nucleoli is not a feature of the histologic picture as observed in the fixed sections. One is impressed by the rapidity of growth in tissue culture as compared with the relatively benign look of the tumor in the histologic section. It is important to correlate this mode of outgrowth in tissue culture with what may happen if cystadenomas of the ovary are ruptured at surgery. It is well-known that if this happens the tumor tends to seed itself over the abdomen and thus make for a very grave prognosis. A phenomenon comparable to this has occurred here in tissue culture with the rather avid growth of these epithelial cells once they got out into an unrestricted favorable medium. Something inherent in the character of the cyst must cause a slow, restricted growth of the tumor in its original site.

2. Tissue from patient A. B.—JSH No. 975–G, a 53-year-old white

woman, with a diagnosis of *papillary carcinoma* of the ovary, was used for cultures to be compared with those from patient A. R. with a diagnosis of *papillary cystadenocarcinoma* of the ovary.

Sections through the tumor showed very marked papillary formation, with some connective-tissue strands running through its substance, and with long, delicate stalks of tumor cells extending out from papillary bases (fig. 38). The individual cells showed a considerable amount of variation in size, shape, and staining characteristics (fig. 39). Mitotic figures were fairly frequent, and there were some bizarre giant-cell forms present. From its histology, the impression is that this tumor was of high-grade malignancy, characterized by marked anaplasia of the cells. However, the one feature that detracts from this interpretation was its maintenance of a papillary pattern.

Thirty roller slides, each carrying 3 explants, were set up in the usual manner. The fluid nutrient contained 50 percent pleural fluid (W).

In contrast with cultures from all of the other patients in this series, outgrowth of epithelial sheets was observed within 48 hours. Stained preparations showed that the epithelium emigrated not only from the margin of explants but also from tiny cellular clusters at some distance from the main mass of tissue. Mitoses were not encountered at this time. The tiny epithelial islands showing vigorous outgrowth of cells within the 48-hour period of incubation are believed to provide an index of the virulence of this particular tumor correlated with the report of high-grade malignancy made from sections of the surgical specimen. Fibroblasts were not seen at this time, and were never conspicuous during the entire period of culture (14 days). This paucity of stromal reaction may have been correlated with the small ratio of connective tissue in the initial explant or may have resulted from the vigorous activity of the frankly carcinomatous epithelium.

Photographs taken of cultures fixed on the 4th day of incubation show the arrangement of the epithelial sheet in an area where the cells were densely packed (fig. 40), and their arrangement at the edge of the outgrowth (fig. 42). The intensity of proliferation is indicated especially well in figure 41, which shows four mitoses in a limited area. The glandular nature of the growth is evidenced by the tendency for a radial arrangement of cells (fig. 43), and the presence of numerous cytoplasmic vacuoles, some of which attained very considerable size, can be seen in figures 40 and 42.

Individual cells and small clusters found at some distance from explants were photographed on the sixth day to show a continuous series, indicating the probability that epithelial islands were being organized from individual cells (figs. 44–48). The nuclei of these cells were large, deeply chromatic, and showed a marked tendency to variation in size and outline. Moreover, nuclei were frequently deeply lobulated (fig. 44) and sometimes associated with small, spherical, nuclear fragments (fig. 46). Probably some nuclei were undergoing disintegration (fig. 47), which may account for the presence of debris in the cytoplasm. The cytoplasm stained lightly

and showed conspicuous, irregular, clear areas (fig. 46) suggestive of secretory activity.

At this time (6 days), the cells within the sheets adjacent to the explant (fig. 50) showed the same features as those described for the isolated cell clusters. The cells in the marginal zone showed marked pleomorphism. For example, the lobulation of nuclei shown in figure 49 is suggestive of amitosis. There were frequent mitoses, and the rapid proliferation of the epithelial sheets may have been aided by the amitotic process. Vacuolated areas in and about the various nuclei are especially well seen in figures 49 and 51. This preparation also shows smooth, curved lines running through the nucleus of a cell. This type of nuclear irregularity was very frequent. It has been recorded at higher magnification in figure 52. One is impressed by the solidity of the growth of the tissue culture. It does not tend to form any discernible papillary pattern as observed in the histologic preparations; however, the anaplasia of the cells becomes a very conspicuous feature. Mitotic figures were very frequent. The cells tended to show the same variation in the size and shape of the nuclei as they did in the fixed section. However, in the histologic preparation, the distortion was accentuated, which may have been due, in part, to the fixation of the tissue. Some of the cells grown in tissue culture showed the same irregular distortion that was observed in the fixed section. Regarding the nuclear-cytoplasmic ratio in general in the fixed section, the nucleus was quite large. The cytoplasm was a relatively inconspicuous feature of the cell and was somewhat irregular and vacuolated. This pattern was maintained in tissue culture. Here again, then, a rather close correlation was found for the behavior of the tumor in tissue culture and its histologic features as observed in the fixed section.

DISCUSSION

In an extensive study dealing with the growth of cells *in vitro*, Gey and Gey (*8*) reported that human tissue was not difficult to handle and that it was their impression that most types of human sarcomas could be maintained indefinitely. The illustrations in this paper indicate similarities between histologic preparations and the pattern of outgrowth in culture.

The literature on the cultivation of ovarian tissue is not extensive. Chlopin (*9*) states that on the basis of tissue cultures, Sertoli cells of the testis, as well as the follicular cells of the ovary, appear to be closely related to mesothelial cells. Vercesi and Guercio (*10*) suggest that the injection of gonadotrophic hormones in rabbits stimulates a richer outgrowth from the ovary, but the cultures that they illustrate appear to consist principally of fibroblasts. In a brief note, Zondek and Wolff (*11*) state that the cultivation of human ovary is difficult, especially with respect to epithelial cells, but that they were successful in maintaining cultures for several weeks. The work was undertaken with the aim of obtaining transplantable tissue for deficiencies. Using a fetus of the eighth month, these workers cultivated the germinal layer including typical granulosa cells. More recently, Payne and Meyer (*12*) reported

that ovarian tissue in roller tubes produced a thick capsule of fibroblasts, but their study was primarily concerned with a method for testing endocrine function.

While the technique utilized in the present study did not reveal epithelial outgrowth from the adult human ovary free of neoplastic tissue, no special effort was made to select areas that might be particularly rich in epithelial cells.

SUMMARY

Roller-tube plasma-clot cultures on 12 by 50 mm. No. 1 cover glasses, utilizing a single feeding with 3 ml. of fluid medium consisting of 50 percent pleural or ascitic fluid, 45 percent Gey's balanced salt solution, 5 percent chick-embryonic extract and 1,000 units per ml. of penicillin, were used in making more than 1,400 preparations of human ovarian explants.

Results obtained on five nonneoplastic ovarian tissues, a Brenner tumor, and two papillary carcinomas of the ovary are described.

With the tissue-culture technique employed, no epithelial sheets were obtained from 750 explants in the nontumor series. The Brenner tumor gave rise to excellent outgrowth of epithelial cells but without conspicuous mitotic activity, except in fibroblastic elements.

Proliferation from epithelial sheets was especially marked in cultures of a papillary cystadenocarcinoma and a papillary carcinoma. The growth potential *in vitro* for these tumors is evidently well correlated with the grade of their virulence as determined from studies of the histologic section. This correspondence is also true for the basic cellular arrangement, as well as the staining properties and form of the nuclei.

In preliminary experiments, the addition of various sterols to the culture medium showed this method of study to be promising, especially when hormones were added directly into perfusion chambers in which cells of ovarian origin were cultivated and records were being made with phase contrast time-lapse cinematography.

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PLATE 147

Outgrowth of spindle cells from explants of nonneoplastic ovarian tissue cultivated *in vitro*. The scale of magnification for all photographs is indicated on figure 1. The cultures represented were fixed in methyl alcohol (refined) on the 10th day of incubation and stained with Jacobson's method.

FIGURE 1.—Patient N. W., normal ovary.

FIGURE 2.—Patient L. C., normal ovary.

FIGURES 3 and 4.—Patient M. E., follicular cysts of the ovary.

FIGURE 5.—Patient A. C., normal ovary.

FIGURE 6.—Patient W. J., normal ovary.

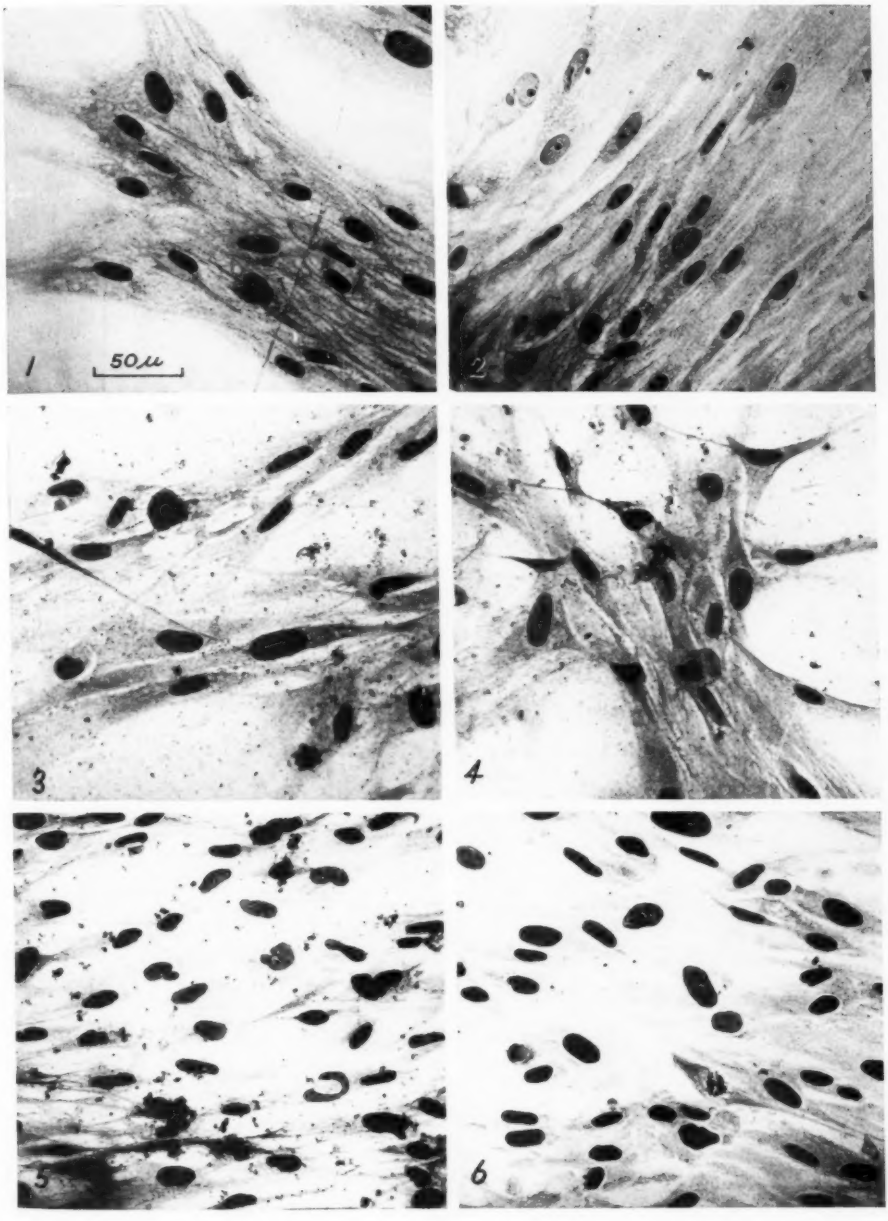

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PLATE 148

FIGURES 7 and 8.—Histologic section of Brenner tumor (patient A. M.). Section at low power (fig. 7) shows epithelial nests resting in a fibrous connective-tissue stroma. Figure 8 represents an individual epithelial cluster.

FIGURES 9–13.—Tissue cultures of the tumor shown in figures 7 and 8. The scale of magnification is shown on figure 9. Spindle cells of the fibroblastic type with frequent mitoses fixed on the 6th day of cultivation (figs. 9 and 10). Epithelial sheet with densely staining nuclei fixed on the 6th day of cultivation (fig. 11). Dense mat of spindle fibers with frequent mitoses as seen in areas close to the explants between 8 and 27 days (figs. 12 and 13).

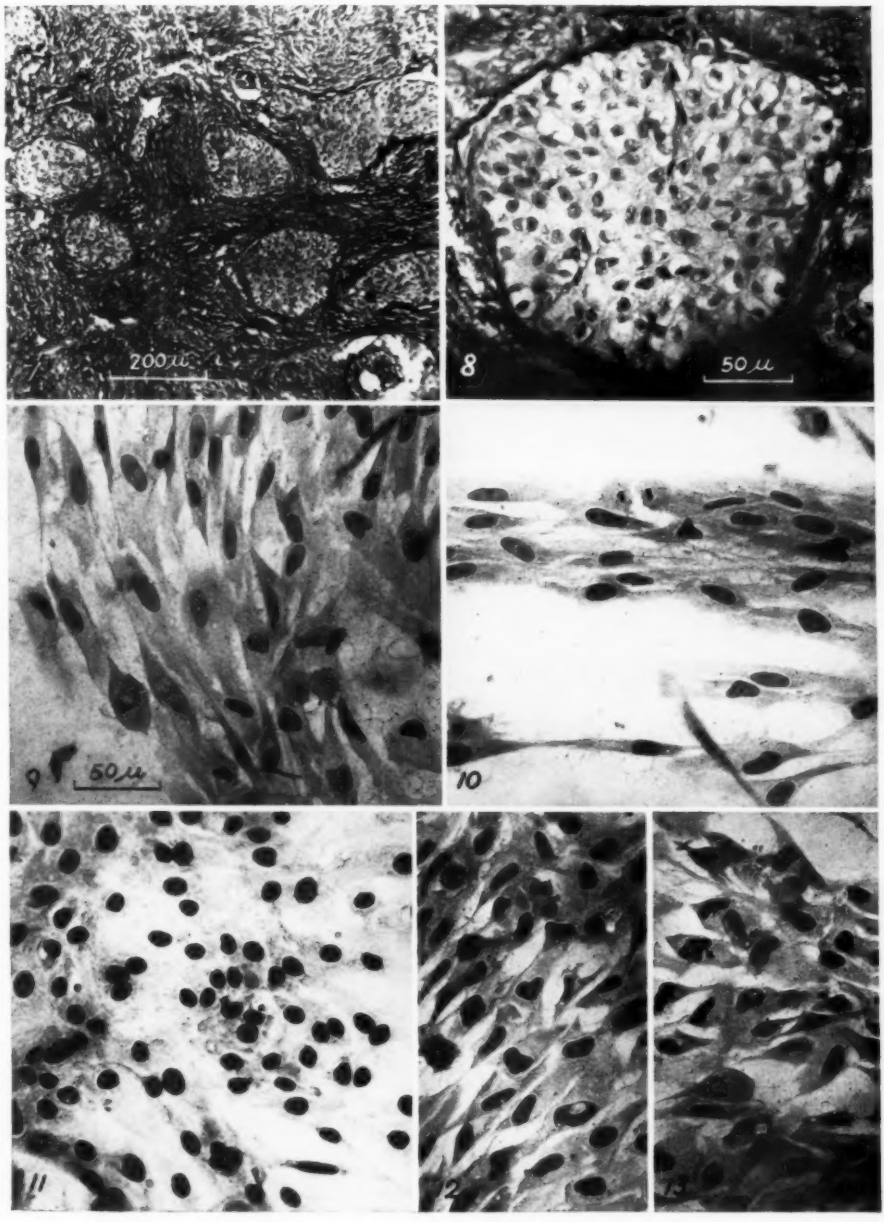
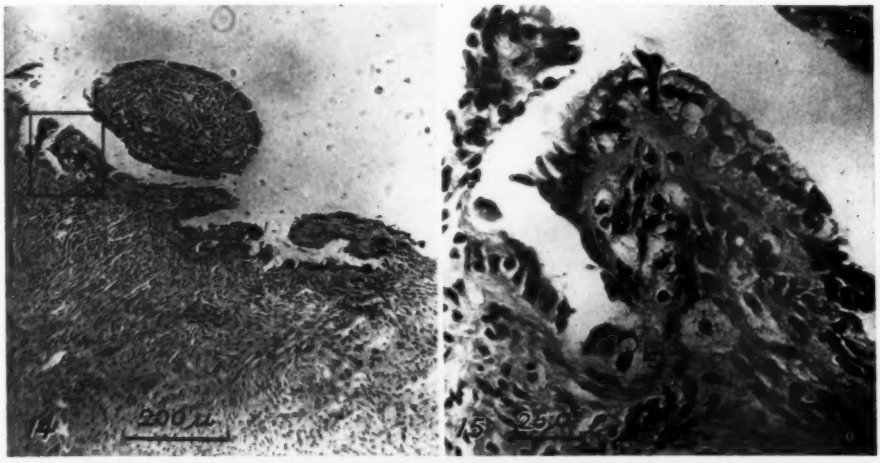

200μ
8
50μ
9
50μ
10
11
12
13

PLATE 149

FIGURES 14 and 15.—Histologic section of a papillary cystadenocarcinoma of the ovary (patient A. R.). Low-power photograph (fig. 14) of papillary structures within cystlike spaces. Figure 15 illustrates area of inset shown in figure 14 at higher magnification, showing the cuboidal and columnar epithelium lining of the cystic wall.

FIGURE 16.—Living culture from the ovary represented in figures 14 and 15, photographed on the 5th day of incubation with an 8-mm. dark M phase contrast objective showing pinocytosis (p), undulating border (b), and mitoses (m).


14
200 μ
15
250 μ

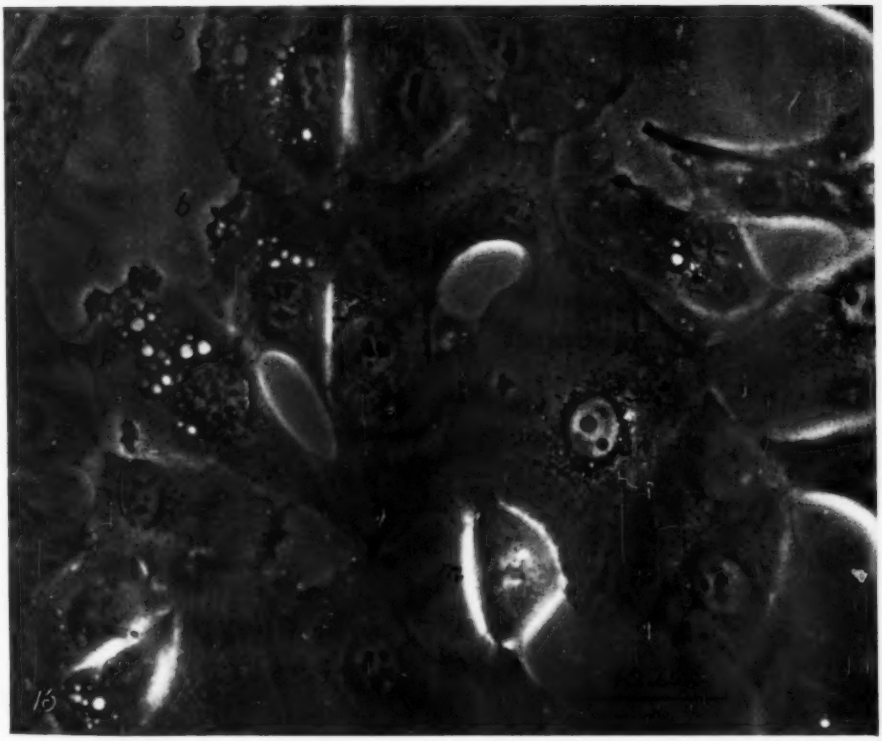

16

PLATE 150

FIGURES 17–20.—Living cultures from the ovary represented in figures 14 and 15, photographed on the 5th day of incubation with a 1.9-mm. dark phase objective, showing the sharp outline of nuclear membranes, prominent nucleolar-associated chromatin and perinuclear filamentous mitochondria and granules. The scale of magnification for all photographs is indicated on figure 17.

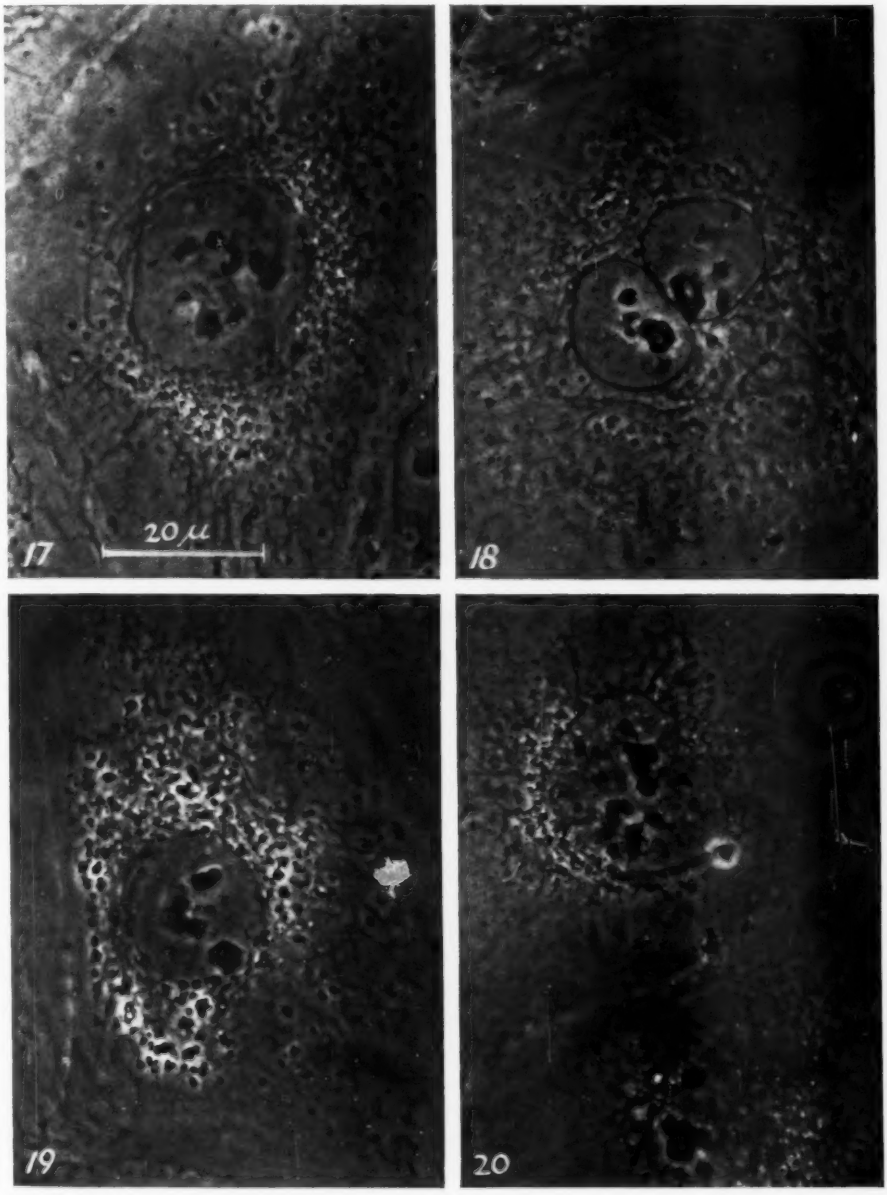
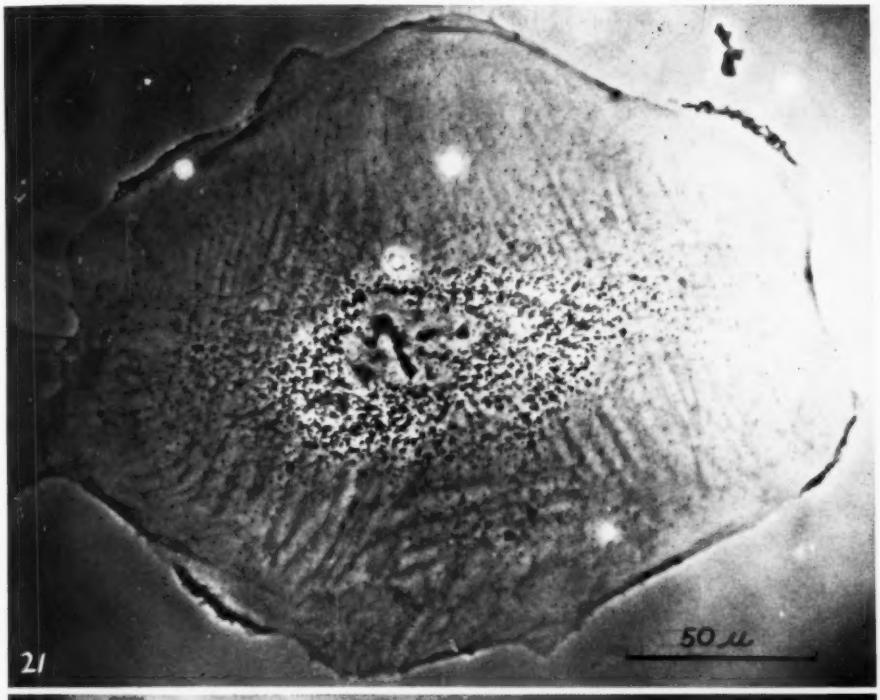

17
20 μ
18
19
20

PLATE 151

FIGURES 21 and 22.—Individual giant cells in 14-day cultures of the papillary cystadenocarcinoma of the ovary represented in histologic section in figures 14 and 15. The photographs were made of the living cells, using an 8-mm. dark M phase contrast objective. The scale of magnification for both photographs is shown on figure 21. Both cells show dense chromatin-associated nucleoli and a heavy concentration of perinuclear granules. The cytoplasm was characterized by conspicuous fibrillar systems which may represent a temporary skeletogenous framework. The marginal undulating membrane and a point of contact with an adjacent cell are particularly well seen in figure 21. The cell shown in figure 21 was used in a perfusion chamber for studying the effect of testosterone and estrone with time-lapse cinematographic technique.

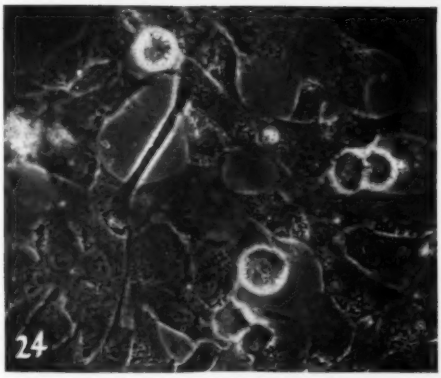

50 μ
21

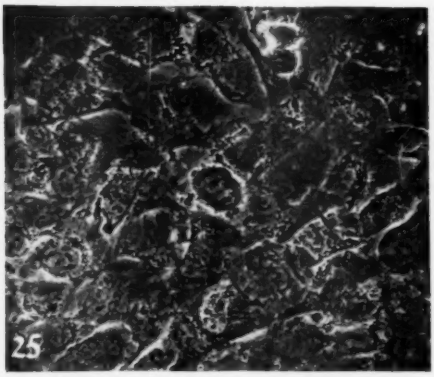
22

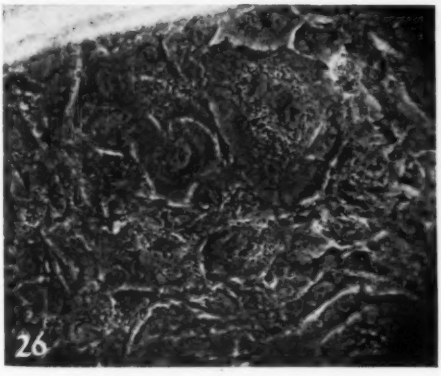
PLATE 152

FIGURES 23–27.—Explant of the papillary cystadenocarcinoma of the ovary, represented in histologic section in figures 14 and 15, cultivated in a medium containing Na-estrone SO_4—12.5 gamma per ml. (Ayerst, McKenna & Harrison). Preparation stained with Jacobson's technique (fig. 23) on the 11th day of incubation showing an enormous spread of uniformly arranged epithelial cells. Photographs of the living culture (figs. 24–27) taken on the 9th day with an 8-mm. dark M phase objective showing variations in cell size, number of nuclei, and mitotic activity. The scale of magnification is shown on figure 24.

23
2 mm


24


25


26

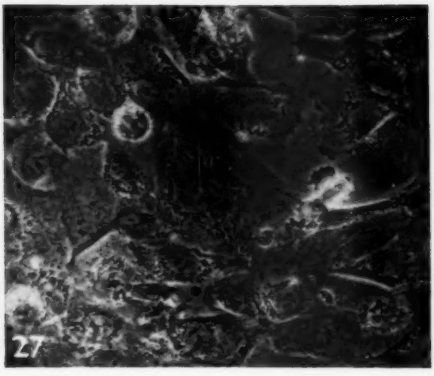
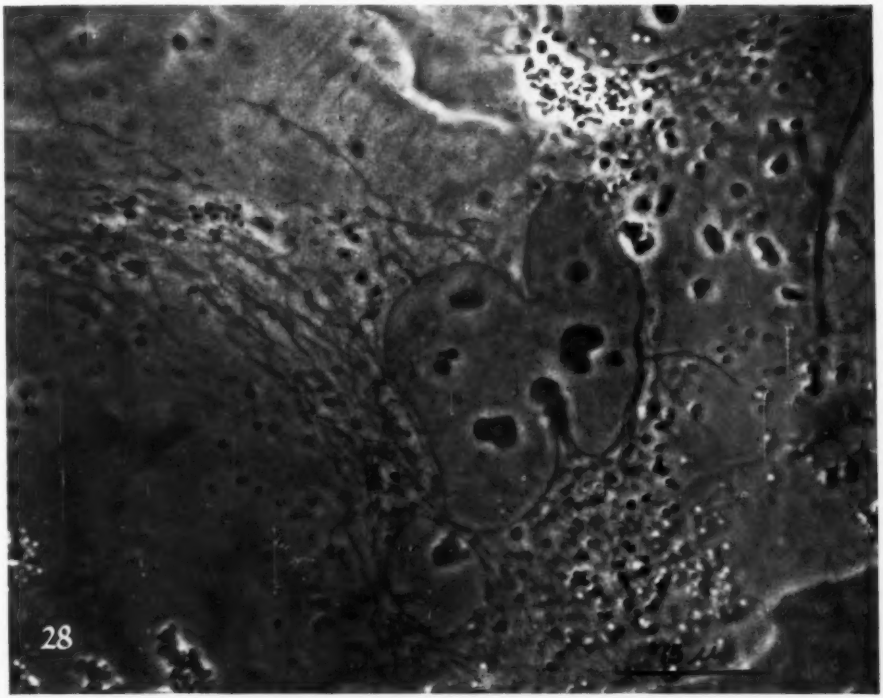
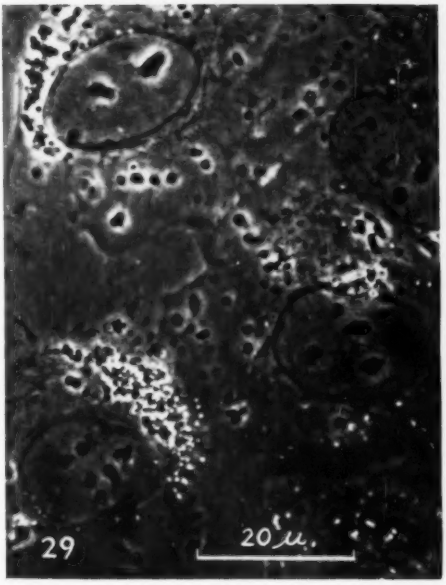

27

PLATE 153

FIGURES 28–30.—Cytologic details in living cells of the culture shown in figure 23. The scale of magnification for photographs 29 and 30 is shown on figure 29. A cell with a small spherical and a large, irregular nucleus with long, filamentous mitochondria (fig. 28). Area in the zone of closely packed cells (fig. 29) showing the presence of relatively small nuclei as compared with that shown in figure 30 from another portion of the outgrowth. Giant nucleus with conspicuous nucleolar-associated chromatin (fig. 30).


28


29
20μ.

30

PLATE 154

FIGURES 31–33.—Cytologic detail in living cells of the culture shown in figure 23. The scale of magnification for all photographs is shown on figure 31. Arrangement of cells in more than one layer showing the relation of the nucleus, an aggregation of small spherical droplets constituting a juxtanuclear zone and long, filamentous mitochondria (fig. 31). Junction between two epithelial cells showing fine strands of cytoplasm (fig. 32). The photograph (fig. 33) represents a typical juxtanuclear zone, sharply defined with phase contrast optics by intense halation of spherical droplets. Note portions of filamentous mitochondria radiating from the juxtanuclear zone as well as in an adjacent cell. An irregular line in the right-hand third of the photograph suggests the position of the overlapping margin of the cell.

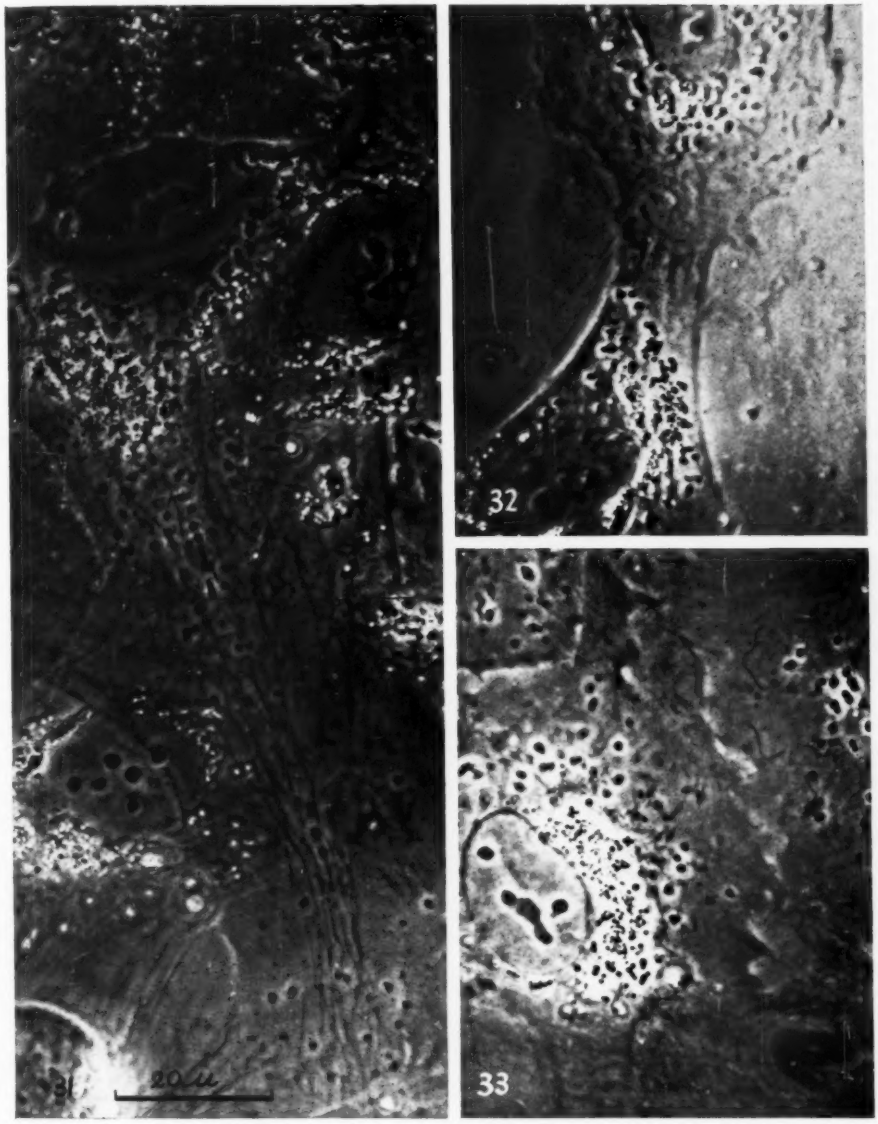

31
20 μ
32
33

PLATE 155

FIGURES 34–37.—Cellular arrangement in the epithelial sheet shown in figure 23, following staining with Jacobson's method. The scale of magnification for all photographs is shown on figure 34. Arrangement of cells in a compact zone showing conspicuous variation in nuclear size (fig. 34). Looser arrangement of cells near the margin of the outgrowth (fig. 35). Pattern of cells at the margin of the outgrowth (fig. 36). Variations in size and outline of nuclei frequently encountered in the epithelial sheet (fig. 37).

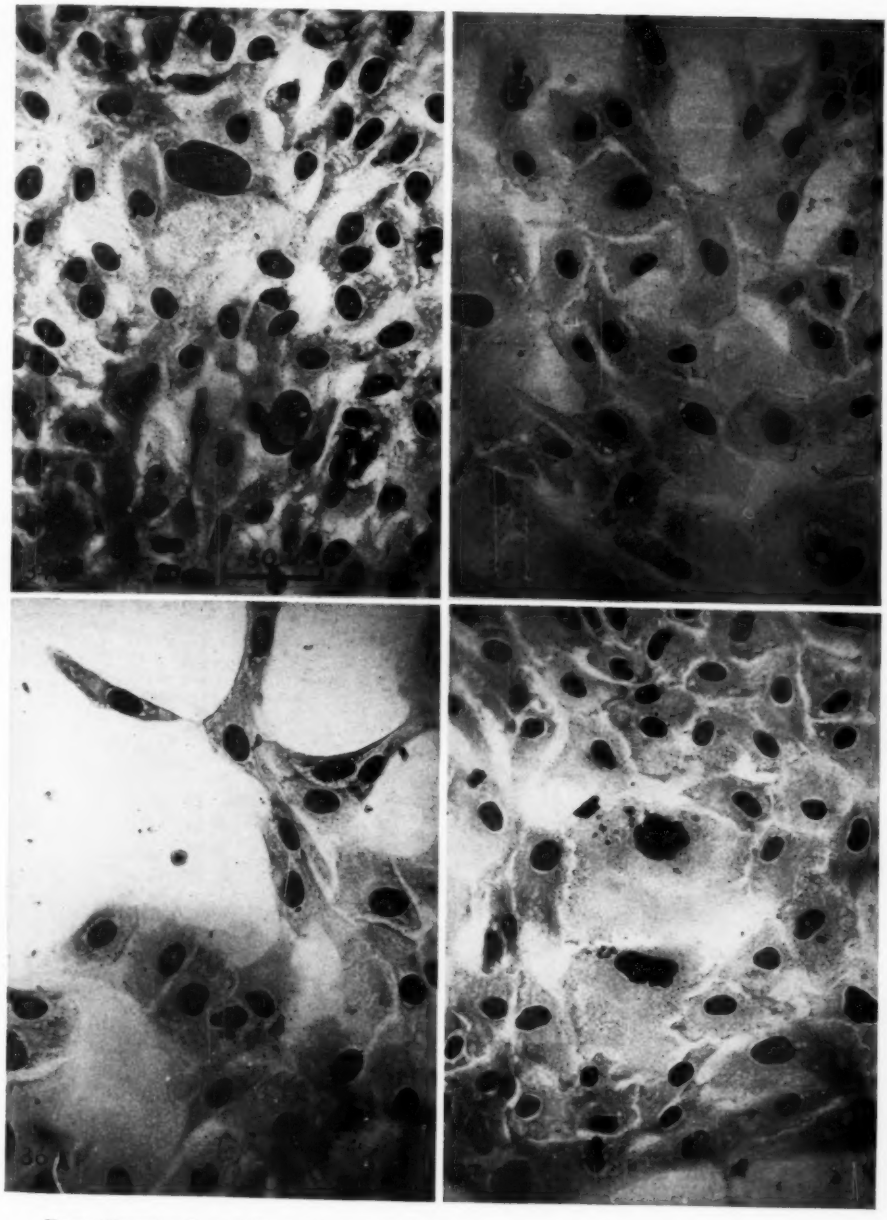


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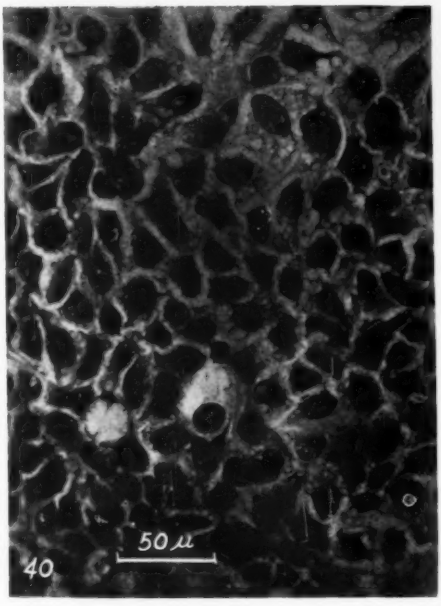
PLATE 156

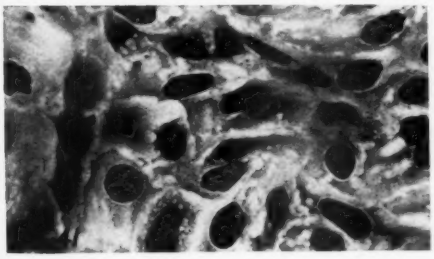
FIGURES 38 and 39.—Histologic section of a papillary carcinoma of the ovary (patient A. B.). Figure 38 shows the typical papillary structure of the tumor. Figure 39 represents a mass of epithelial elements with evidence of inconspicuous strands of stromal material.

FIGURES 40–42.—Tissue-culture outgrowth from explants of the tumor shown in figures 39 and 40 after 4 days of incubation. The scale of magnification is shown on figure 40. Compact arrangement of cells (fig. 40) in the epithelial outgrowth close to the explant. Mitotic activity in the midportion of the outgrowth (fig. 41). Margin of the outgrowth showing mitotic activity and conspicuous vacuoles (fig. 42).

38
100 μ

39


50 μ
40



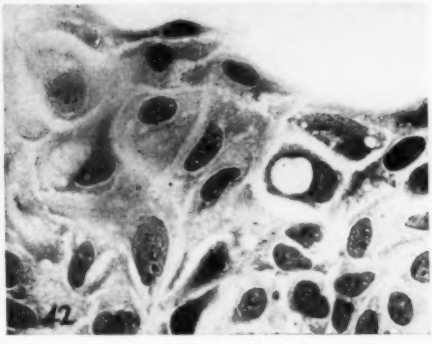

42

PLATE 157

FIGURES 43–48.—Cells in tissue culture from the papillary carcinoma of the ovary represented in figures 38 and 39, stained with Jacobson's method. The scale of magnification for all photographs is shown on figure 43. Pattern of cells at the margin of the outgrowth after 4 days of incubation, showing a tendency to form an acinar arrangement (fig. 43). Photographs (figs. 44–48) arranged to suggest a possible series of stages in the formation of an epithelial sheet from an isolated malignant cell. Such islands of malignant elements were commonly scattered over the entire slide.

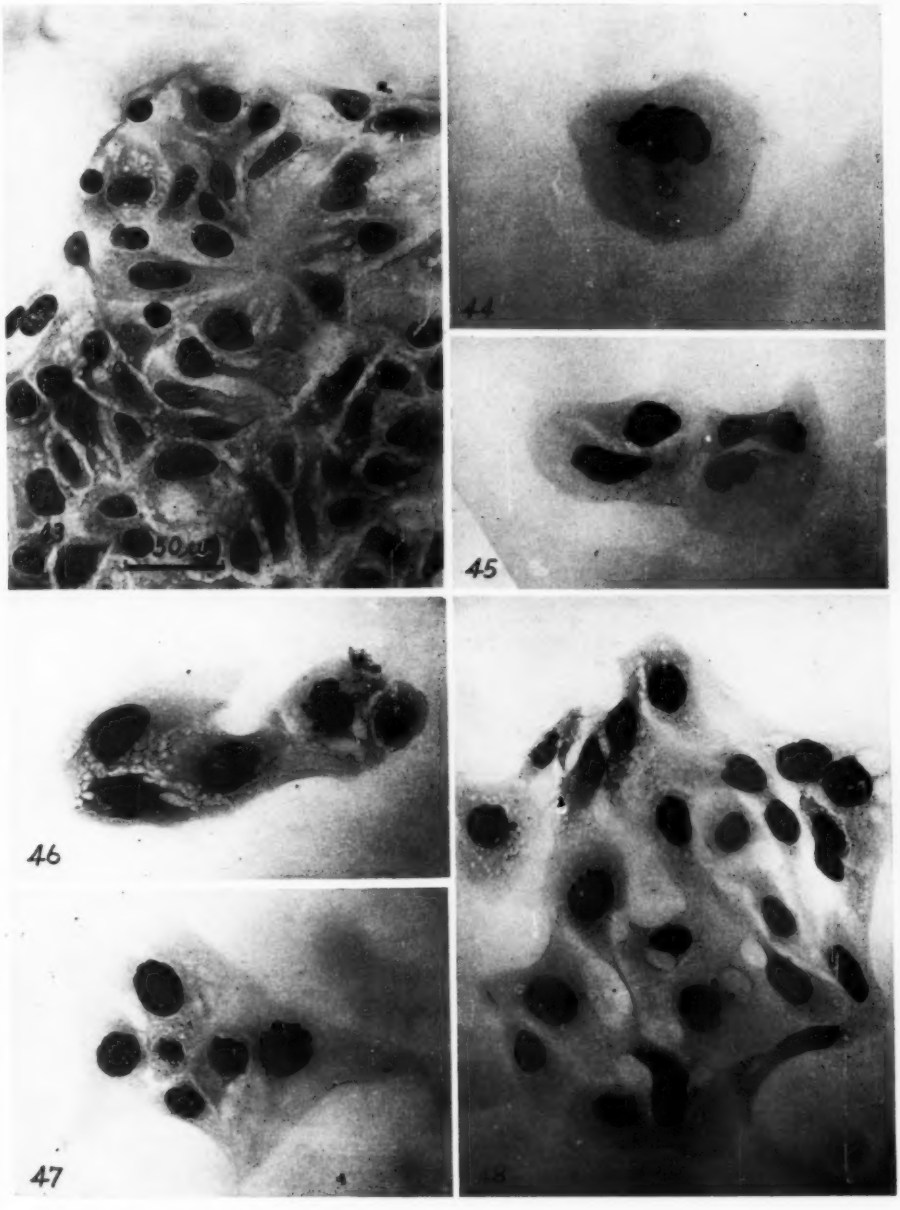
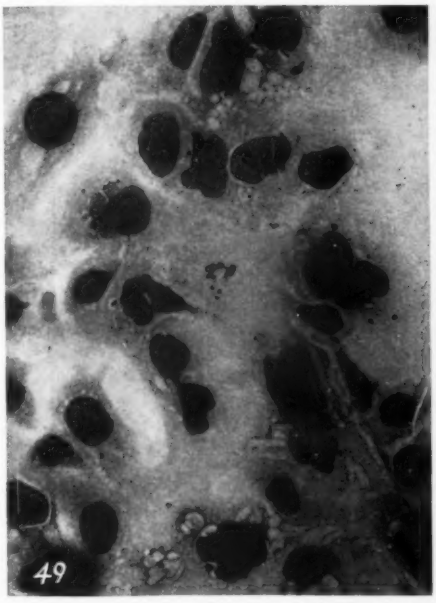
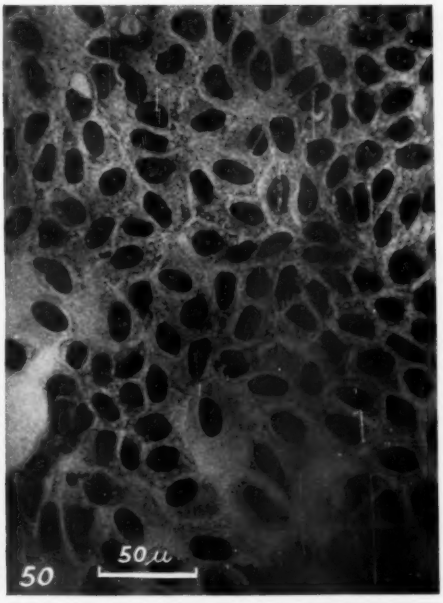

43
50μ
44
45
46
47

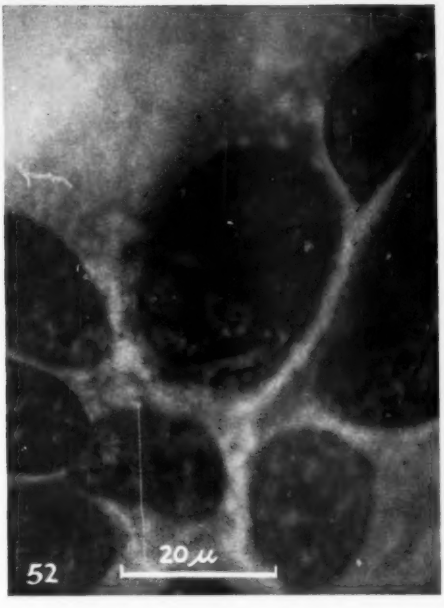
PLATE 158

FIGURES 49–52.—Cells in tissue culture from the papillary carcinoma of the ovary represented in figures 38 and 39. Cultures fixed on 6th day and stained with Jacobson's. Scale of magnification for figures 49–51 is shown on figure 50. Cells near the margin of the explant (fig. 49) showing nuclear pleomorphism and cytoplasmic vacuolization. Compact arrangement of nuclei of epithelial outgrowth in the area adjacent to the explant (fig. 50). Epithelial cells at the margin of the culture showing multinucleation (fig. 51). Characteristic patterns in the nuclear membranes frequently encountered in epithelial cells at higher magnification (fig. 52).


49


50
50μ

51


52
20μ

STUDIES ON THE "CYTOTOXIC" PROPERTY OF MAMMARY TUMOR AGENT ANTISERUM [1]

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The mouse mammary tumor agent is highly antigenic. When injected into rabbits or rats it stimulates the production of immune bodies which will inactivate filtrate or centrifugate preparations of the mammary tumor agent *in vivo* and *in vitro* (*1*, *2*). Attempts to demonstrate neutralizing antibodies to the mammary tumor agent, in mice, under varying conditions, have met with failure (*3*), despite efforts to follow closely the experimental procedures used in producing neutralizing antibodies in the rabbit and rat. Complement-fixing antibodies (*4*) and precipitins (*5*) specific for the mammary tumor agent have also been reported.

In addition to these specific antibodies, an immune serum prepared in rabbits against centrifugates of mouse mammary tumors has been reported that specifically and completely inhibited the growth of mammary tumor cells incubated with the serum (*6*). A cell suspension obtained from a mammary tumor in a strain C3H mouse was mixed with so-called "cancer antiserum" obtained in rabbits by injections of a high-speed centrifugate of homogenized mammary tumor tissue from strain C3H mice. Following incubation at room temperature for 3 hours and at 7° C. for 3 hours, the preparation was inoculated intraperitoneally into test mice apparently free of the mammary tumor agent. Controls consisted of mixing the tumor-cell suspension with saline, normal tissue antiserum and normal rabbit serum, respectively. The inoculated mice were then observed for tumors for a period of 60 days. During this period of observation, tumors developed rapidly in all of the saline-control mice and in none of the mice receiving the mixture of tumor cells and cancer antiserum. However, it is to be noted that some interference was obtained in the two other sets of controls as determined by the number of "takes" and the rate of growth of the transplanted cells. Recently, Imagawa, Bittner, and Syverton (*7*) have published preliminary data indicating a marked inhibitory effect of guinea-pig antisera prepared against tissues containing the mammary tumor agent but no inhibition by normal guinea-pig serum. Samples of rabbit antiserum, prepared against tissues containing or freed of the agent, showed, on the other hand, a constant inhibitory effect, apparently not related to the presence of the mammary tumor agent.

[1] Received for publication February 20, 1951.

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It is the purpose of this report to present findings, in controlled experiments, of the effects of antisera on the growth of mouse mammary tumor cells as determined by transplantation. The antisera were prepared in the rabbit against normal lactating mouse mammary tissue and mammary tumor tissue containing the agent or freed of it by foster nursing.

MATERIALS AND METHODS

We have attempted to select the tissues used as the sources of antigens, either mammary tumor or normal lactating mammary gland, so that any observed antigenic differences would not be related to 1) differences in the genetic constitution of the tissues, or 2) malignancy *per se*. Consequently, tumor tissue with the mammary tumor agent has always been compared with tumor tissue freed of the agent and normal lactating tissue containing the agent with the normal counterpart freed of the mammary tumor agent. Closely related mice of strain C3H, with the mammary tumor agent, and strain C3Hb, freed of the agent (*8*), have been used throughout as a source of tissues.

The mammary-tumor incidence of breeding females of strain C3H, as reported by Heston *et al.* (*8*), is 97 percent at an average age of 7.8 months, and of strain C3Hb breeding females, 38 percent, at an average age of 20.3 months. Virgin females, however, of strain C3Hb have a low incidence of mammary tumors, 4 percent, at an average age of 24.2 months. Standard biologic tests for the presence of the mammary tumor agent in 24 spontaneous mammary tumors of C3Hb mice have been negative, indicating the absence of the agent (*8*). Mammary tumor tissues used as antigens have been obtained from the strains C3H and C3Hb mice described. Lactating mammary tissues deprived of the agent, used as antigens in the present experiments, were obtained from another subline of C3Hb mice described later.

Adenocarcinoma with Mammary Tumor Agent

Mammary tumor L 1221, a typical adenocarcinoma, has been used throughout this study as a source of antigen containing the mammary tumor agent. This tumor arose in female C3H, No. 2 of the F_{15} generation [2] at 7 months of age. It has been carried in serial transplant in C3Hb mice only, that is in mice apparently free of the agent. Biologic tests for the presence of the agent have been made at the 5th, 10th, and 20th transplant generations. A 20 percent Berkefeld filtrate in saline was injected, 0.2 cc.. intraperitoneally, into appropriate test mice, strain B alb C (C)[3] at 2–4 weeks of age. Seventeen mammary tumors were induced in 31 test mice at an average age of 9.2 months, with approximately equal distribution of tumors at each transfer tested. Transplant generation 19 of tumor L 1221 was used as the source of antigenic material in experiment

[2] Obtained originally by Dr. W. E. Heston from Dr. H. B. Andervont of this Institute. F_{15} indicates 15 generations of brother × sister matings since that time.

[3] The mammary tumor incidence in the breeding colony of our strain C (B alb C) females has been less than 1 percent.

1, transplant generation 29 was used as antigen material in experiment 4 and transplant generation 38 was used in experiments 7 and 8.

Adenocarcinomas Without the Mammary Tumor Agent

Tumor MT 7B, a typical mammary adenocarcinoma, arose spontaneously in female C3Hb No. 1148 of generation F_6 [4] at 17 months of age. This particular line of C3H mice had been deprived of the mammary tumor agent by foster nursing of young following their removal by caesarean section. Appropriate biologic assays for the agent in numerous mammary tumors of this line have been consistently negative (*8*). Tumor MT 7B was tested for the mammary tumor agent by the method described above. Eighteen strain C (B alb C) test mice have remained tumor free for 18 months following injections of cell-free filtrates of transplant generations 2 and 9 of this tumor. Transplant generation 6 was used as a source of antigenic material in experiment 1, and transplant generation 15 was used in experiment 4.

Mammary tumor MT 8, without the mammary tumor agent, was used as a source of antigen, in experiments 7 and 8. This tumor arose in a strain C3Hb female 16 months of age. Biologic tests for the presence of the agent were accomplished as described above at the second transfer of the tumor in agent-free mice. No tumors have appeared after 17 months in 11 test mice. Tissue from transfer generation 20 was used as antigen.

Mammary Gland Tissue with the Mammary Tumor Agent

Lactating littermate females No. 972 and No. 975 of strain C3H, generation F_{17}, 10 months of age, were used to obtain tissues. Two littermates of these mice had mammary tumors at 11 months of age, and the mother and two sisters of the preceding generation, F_{16}, had mammary tumors at 7, 8, and 8 months, respectively. Antisera produced against these tissues have been used throughout this study.

Mammary Gland Tissue Without the Mammary Tumor Agent

Female C3Hb No. 3043, generation F_3, 6 months of age was used to obtain lactating mammary gland tissue. This animal was 3 generations removed from females No. 972 and 975 of strain C3H described above. The F_1 generation was removed from the mother by caesarean section and foster-nursed by a strain C57 black female. Brother $\times$ sister matings have continued since, through the F_4 generation, and only 2 (4 percent), mammary tumors have been observed in 50 breeding females in this particular C3Hb line.

Preparation of Antigen

Two methods were used for the preparation of the antigens. The method described by Green (*6*) was followed exactly in the preparation

[4] This tumor was obtained from Dr. W. E. Heston. F_6 designates 6 generations of brother $\times$ sister matings following foster-nursing of a strain C3H litter on strain C57 black.

of one group of C3H and C3Hb mammary tumor antigens and referred to as method "G." (See table 1.) The antigen prepared by method G contained 1.4 grams of original tissue per cubic centimeter. A modification of Green's method was used in preparing another group of C3H and C3Hb mammary tumor antigens as well as C3H and C3Hb normal lactating mammary gland antigens. This method is designated method "B" in table 1 and consisted of centrifuging the homogenate at 900 × g for 10 minutes. The supernatant was then centrifuged at 23,000 × g for 1 hour,[5] and the sediment obtained was resuspended in saline, cleared at 900 × g for 2 minutes, and used as the antigen. Material prepared by this method contained approximately 1 gram of original tissue per cubic centimeter.

All procedures were done at 0–4° C., and to avoid repeated freezing and thawing, the antigens were stored in several aliquots at −20° C. One vial was thawed and used for each injection or complement-fixation reaction.

Preparation of Antisera

Two rabbits were injected with each antigen and the antisera from rabbits receiving the same antigen were pooled. A bleeding was done on each rabbit before immunization for use as a control in complement-fixation tests. The antigens prepared by Green's method were injected as Green described, one cubic centimeter intraperitoneally once a week for 5 weeks. Two weeks after the last injection the rabbits were bled from the ear vein.

Rabbits immunized with the mammary tumor antigens prepared by the modification of Green's method received 0.5 cc. of antigen intravenously once a week for 5 weeks and were bled from the ear vein 2 weeks after the last injection.

Two cubic centimeters of the normal mammary gland antigens were mixed with equal volumes of mineral oil and one-tenth volume of Arlacel A and injected subcutaneously. Three weeks later the rabbits were bled from the ear vein. All sera were stored at −20° C. until used.

CYTOTOXIC STUDIES[6]

To test the cytotoxic properties of the sera a cell suspension of transplantable mammary adenocarcinoma L 1221 was used. Nonnecrotic tumor tissue was passed through a tissue press into saline and aspirated through hypodermic needles in decreasing size, No. 18 through No. 27. This material was centrifuged at 600 × g for 10 minutes. After removal of the supernatant fluid and red-cell layer more saline was added and centrifuging repeated. The packed tumor cells were resuspended in saline and mixed in equal volumes with the antisera to be tested to give a final tumor-cell suspension of 12.0 percent. The suspension of cells

[5] According to Barnum *et al.* (9), this technique concentrates the mammary tumor agent in the sediment.

[6] The term cytotoxic is used here because of the use of this term in the earlier literature by Green. It refers to inhibition or retardation of the growth of mammary cancer cells. Histologic studies to determine the manner of inhibition of growth have not been accomplished.

and antisera was incubated for 1 hour at 37° C. and mixed 2–3 times during the period of incubation. In experiments 1, 2, and 3, 0.2 cc. of the suspension was inoculated subcutaneously into C3Hb mice of both sexes.

Several variations of this method were used in separate tests. All of the cell suspensions were prepared in the same way, and the same tumor, L 1221, was used. In experiment 4, table 1, a 6 percent cell suspension was prepared in straight serum without dilution. This was done by making up the 6 percent cell suspension in saline and then sedimenting the cells in a centrifuge at 900 × g for 10 minutes. The saline was removed by aspiration and replaced by the same volume of serum. The cells were resuspended and incubated at 37° C. for 3 hours, followed by 3 hours in the ice box at 6° C. C3Hb mice of both sexes were injected subcutaneously with 0.1 cc. of this tumor cell and serum mixture.

Another variation consisted of a 12.0 percent cell suspension in 5 parts of serum to 1 part saline. This mixture was incubated at 37° C. for 6 hours and 0.1 cc. was injected subcutaneously into each C3Hb mouse (experiment 5).

In another series, experiment 6, 0.6 percent tumor cells in straight antiserum was incubated at 37° C. for 6 hours. Of this preparation, 0.1 cc. was injected into each C3Hb mouse.

In experiments 7 and 8, 0.5 percent tumor cells was made up in 1 part of saline and 5 parts of serum and incubated at 37° C. for 3 hours, followed by 3 hours at 6° C. Each C3Hb mouse was injected subcutaneously with 0.1 cc. of this mixture.

In all of the experiments the tumor cell and serum preparation was mixed thoroughly every 20 to 30 minutes during the period of incubation.

Complement-fixation tests were performed on all sera before and after immunization to test for the presence of complement-fixing antibodies. The sera were tested against the same antigens used for immunization by a procedure previously described (*10*). In all cases the presence of good complement-fixing antibodies was found in the postimmunization sera.

RESULTS

It can be seen by reference to table 1, experiment 2, that tumor cells of L 1221 incubated with antiserum prepared against lactating mammary gland tissues containing the mammary tumor agent produced 12.5 percent fewer tumors than when incubated with the antiserum against the agent-free lactating tissue. When this antiserum was tested again under identical experimental procedures, after storage for 2½ months (experiment 3), there was found to be no difference between this and the control group.

In experiment 7 an attempt was made, as far as possible, to duplicate the experimental procedures of Green (*6*). When a 0.5 percent suspension of tumor cells, incubated with antiserum prepared against mammary tumor tissue containing the agent, was inoculated into strain C3Hb test mice only 52 percent grew the tumor progressively. The difference between this group and the controls was within the limits of experimental

TABLE 1.—*Transplantation fate of mammary tumor cells of mammary carcinoma L 1221 following incubation with various antisera*

Experiment No.	Antigen used	Antiserum group	Percent cell suspension	Proportions of sera and cell brei used	Number mice	Progressive growth		D±SE [1]	Days at death (range)
						Number	Percent		
I	MT—with agent [2]	Method B	[3] [4] 12.0	Equal	23	22	(95.6)	4.4±4.3	48.2 (36–57)
	MT—agent-free				24	24	(100.0)	P=0.2	47.8 (34–57)
II	LMG—with agent	Method B	12.0	Equal	24	21	(87.5)	12.5±6.9	40.0 (38–41)
	LMG—agent-free				24	24	(100.0)	P<0.05	38.5 (26–40)
III	LMG—with agent	Method B	12.0	Equal	26	26	(100.0)		32.6 (23–35)
	LMG—agent-free				23	23	(100.0)		34.4 (27–35)
IV A	MT—with agent	Method B	6.0	Cells in serum	12	11	(92.5)	7.5±8.6	54.7 (45–69)
	MT—agent-free				11	11	(100.0)	P=0.4	52.4 (38–70)
IV B	LMG—with agent	" "	6.0	" " "	12	11	(92.5)		49.7 (41–61)
	LMG—agent-free				12	11	(92.5)		45.7 (30–70)
V	LMG—with agent	Method B	12.0	1 part brei	15	15	(100.0)		37.4 (26–49)
	LMG—agent-free			5 parts serum	15	15	(100.0)		43.3 (26–61)
VI	LMG—with agent	Method B	.6	Cells in serum	23	22	(95.7)	29.1±11.6	55.9 (40–104)
	LMG—agent-free				21	14	(66.6)	P=0.02	62.0 (40–104)
VII	MT—with agent	Method G	.5	1 part brei	25	13	(52.0)	25.7±13.2	61.9 (34–96)
	MT—agent-free			5 parts serum	27	21	(77.7)	P<0.1	63.3 (42–89)
VIII	MT—with agent	Method G	.5	1 part brei	17	3	(17.6)		56.6 (53–64)
	MT—agent-free			5 parts serum	24	4	(16.6)		54.5 (33–64)

[1] Difference between mean percentages±the standard error of the difference.
[2] MT—mammary tumor tissue; LMG—lactating mammary gland tissue.
[3] A 12.0 percent cell suspension represents approximately 120×10^6 cells; a 6.0 percent suspension represents approximately 60×10^6 cells, 0.6 represents 6×10^6 cells and 0.5 percent suspension represents approximately 5×10^6 cells per cubic centimeter. In experiments number I, II and III, 0.2 cubic centimeter cell suspension was inoculated per animal; 0.1 cubic centimeter was inoculated in the remaining experiments.
[4] Incubation time and temperature for experiments I, II, and III, 37° C. for 1 hour; for experiments V and VI 37° C. for 6 hours; for experiments IV, VII, and VIII, 37° C. for 3 hours followed by 6° C. for 3 hours.

error. When the experiment was repeated using the same sera stored at −20° C. for 2½ months, no differences were then observed between the agent and agent-free groups (experiment 8).

In experiment 6 only 66.6 percent of strain C3Hb mice showed progressive growth of tumor cells which were incubated in antiserum prepared against agent-free lactating mammary gland tissue. In contrast, 22 of 23 test mice, 95.7 percent, progressively grew the tumor cells which were incubated with antiserum prepared against lactating tissue containing the agent. Although there is a strong indication of an inhibitory effect in this experiment, since $P=0.02$, it is opposite to what is expected, assuming the agent antiserum is cytotoxic. Thus, these results must be attributed either to nonspecific inhibition or they represent a sampling error.

In all other experiments, tumor cells incubated with antiserum prepared against tissues with the agent grew equally well as tumor cells incubated with antiserum prepared against tissue freed of the agent.

It should be pointed out that in experiments 6, 7, and 8, the decreased percentages of "takes" is the result of suboptimal numbers of tumor cells employed. This low concentration of tumor cells was intentionally used in the hope of magnifying any possible cytotoxic properties of the antisera.

DISCUSSION

The nature of the immunity response to transplanted neoplasms has long been a matter of interest and a matter of dispute. Russell (*11*) has given a review of the pertinent literature up to 1912 and Woglom (*12*) reviewed the subject up to 1929. Woglom concluded in his review that the type of immunity to transplantable tumors was not related to other known forms of immunity. In the meantime, evidence has been presented from several sources which strongly suggests that the stimuli determining destruction of tumor grafts arise from iso-antigenic differences and the effective substances produced are iso-antibodies [see Gorer (*13*)]. Prior to the work of Gorer, several investigators, notably Lumsden (*14*) and Witebsky (*15*) believed that cancer tissue differed from its normal counterpart and that it was possible to demonstrate modified antigens in the cancerous tissue. These experiments cannot be considered critical because of the failure to control genetic factors and thereby iso-antigenic differences.

The work of Gorer has established that immunity reactions to transplants of normal and tumor tissue have a specific genetic and antigenic basis. His more recent work (*16*) has shown that the presence of protective antibodies against a line of mouse leukemia could be determined by mixing leukemic cells and sera prior to transplantation. A definite retardation in the growth of the localized lymphomatous mass and the infiltrating capacity of the leukemic cells was noted following incubation with antiserum prepared in a line of mice resistant to the leukemic cell transplant. In a later study on the genetic and antigenic basis of tumor immunity, it was shown (*17*) that an antigen responsible for the immune reaction in certain transplantable tumors of the mouse was closely linked

with a gene influencing skeletal growth of the mouse and that this gene was closely linked with an allele of the gene for the blood group antigen, antigen II.

It should be noted again that to date no specific qualitative differences have been shown to exist between normal and tumor tissues as regards the immunity response studied by the method of tissue transplantation. The three instances mentioned by Snell *et al.* (*18*) which would seem to indicate antigenic differences between normal and cancer tissue in inbred animals, may be adequately explained as the result of a change due to mutation in either the cancer tissue or the strain of animal. Although quantitative antigenic differences have been shown to exist (*16*), much care should be used in ascribing too much importance to the difference observed, since in most cases malignant cells have not been compared in transplantation and immunity studies with their normal precursors; for example, malignant lymphoblasts with normal lymphoblasts, fibrosarcoma cells with normal fibroblasts, *etc.*

The problem concerned in the present study is much simplified since it involves a study of the possible cytotoxic property of antisera prepared against a virus-like agent, the mammary tumor agent. Since the presence of this agent can be demonstrated in normal lactating mammary gland tissue (*19*) and since the tissue apparently can be freed of the agent by removing the young by caesarean section and foster nursing on an agent-free line of mice, *e. g.*, strain C57 black, it is possible to produce antisera against tissues which are alike histologically and genetically and differ, only as far as is known, in the presence or absence of the mammary tumor agent. Likewise, since it has been shown that mammary gland adenocarcinomas arise spontaneously in strain C3Hb females freed of the agent (*8*), it is possible to compare antisera produced against tumor tissues which are genetically similar in origin and histologically alike but differ only in the presence or absence of the mammary tumor agent.

In the present study we have attempted to control the materials used as much as possible by using closely related mice of the inbred C3H and C3Hb strains and by comparing response to antibodies as produced against either normal lactating mammary gland tissue or mammary tumor tissue with and without the mammary tumor agent. Heretofore, these obvious control precautions have not always been practiced. The difference in results obtained in our work from those recorded by Green (*6*) require explanation. It is possible that the cytotoxic response shown by Green was the result of differences between the antiserum prepared in one case against tumor tissue, a mammary adenocarcinoma and against normal tissue, the lactating mammary gland. Normal rabbit serum, in his experiment, also produced a slight retarding effect suggesting quantitative differences in the sera used. Since the fate of transplanted tumor cells was not determined beyond a 60-day period, this point cannot be established definitely from his data. It is pertinent to note also that the experimental and control tissues used as antigens were not obtained from genetically similar animals. Tumor tissue was obtained from strain

C3H mice and normal lactating mammary gland tissue from ZBC mice, which are backcross mice using strain C3H as one of the parents. A difference in response of tumor cells incubated with antisera prepared against tissue of 2 different inbred strains of mice, strains C3H and dba, was noted previously (*20*). A definite inhibition of growth in transplantation was observed in 3 different transplantable mammary tumors when strain dba tissues, with or without the agent, were used, while in contrast no inhibition of growth was found when tissues of strain C3H mice, with or without the agent, were used as source of antigen. This would suggest that nonspecific inhibition of growth of mammary tumor cells may occur under certain experimental conditions.

SUMMARY

Under the conditions of the experiments reported here, there were found to be no consistent differences in the percentage of progressively growing tumors or in the mean age at death of mice inoculated with a transplantable mammary adenocarcinoma following incubation of tumor cells with antisera produced in rabbits against mammary tumor tissues containing or freed of the mammary tumor agent nor against normal lactating mammary gland tissues containing or freed of the agent.

The results obtained are discussed in relation to reported experiments which indicated a "cytotoxic effect" on mammary tumor cells of the mammary tumor agent antiserum.

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A METHOD OF ESTIMATING COMPARATIVE RATES FROM CLINICAL DATA. APPLICATIONS TO CANCER OF THE LUNG, BREAST, AND CERVIX [1]

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A frequent problem in epidemiological research is the attempt to determine whether the probability of having or incurring a stated disease, such as cancer of the lung, during a specified interval of time is related to the possession of a certain characteristic, such as smoking. In principle, such a question offers no difficulty. One selects representative groups of persons having and not having the characteristic and determines the percentage in each group who have or develop the disease during this time period. This yields a true rate. The difference in the magnitudes of the rates for those possessing and lacking the characteristic indicates the strength of the association. If it were true, for example, that a very large percentage of cigarette smokers eventually contracted lung cancer, this would suggest the possibility that tobacco is a strong carcinogen.

An investigation that involves selecting representative groups of those having and not having a characteristic is expensive and time consuming, however, and is rarely if ever used. Actual practice in the field is to take two groups presumed to be representative of persons who do and do not have the disease and determine the percentage in each group who have the characteristic. Thus rather than determine the percentage of smokers and nonsmokers who have cancer of the lung, one determines the percentage of persons with and without cancer of the lung who are smokers. This yields, not a true rate, but rather what is usually referred to as a relative frequency. Relative frequencies can be computed with comparative ease from hospital or other clinical records, and in consequence most investigations based on clinical records yield nothing but relative frequencies. The difference in the magnitudes of the relative frequencies does not indicate the strength of the association, however. Even if it were true that there were many more smokers among those with lung cancer than among those without it, this would not by itself suggest whether tobacco was a weak or a strong carcinogen. We are consequently interested in whether it is possible to deduce the rates from knowledge of the relative frequencies.

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A GENERAL METHOD

To fix our ideas we may illustrate how the general problem can be attacked with some data recently published by Schrek, Baker, Ballard, and Dolgoff (*1*). They report that 77 percent of the white males studied, aged 40–49, with cancer of the lung, smoked 10 or more cigarettes per day, while only 58 percent of a group of white males, aged 40–49, presumed to be representative of the non-lung-cancer population, smoked that much. Can we estimate from these data the frequency with which cancer of the lung occurs among smokers and nonsmokers?

Denote by p_1 (=0.77) the proportion of smokers among those with cancer of the lung, by p_2 (=0.58) the proportion of smokers among those without cancer of the lung, and by X the proportion of the general population that has cancer of the lung during a specified period of time. We may then summarize the relevant information for the general population in a two-by-two table showing the proportion of the population falling in each of the four possible categories.

Characteristic	Having cancer of the lung	Not having cancer of the lung
Smokers	p_1X	$p_2(1-X)$
Nonsmokers	$(1-p_1)X$	$(1-p_2)(1-X)$
Total	X	$1-X$

One can now compute that the percentage of the general population that smokes is $p_2+X(p_1-p_2)$, that the proportion of smokers having cancer of the lung is:

$$(1)\quad p_1X/[(p_2+X(p_1-p_2)].$$

Similarly, the proportion of nonsmokers having cancer of the lung is

$$(2)\quad (1-p_1)\,X/[(1-p_2)-X\,(p_1-p_2)].$$

Formulas (1) and (2) yield the true rates we seek.

Given the appropriate data, formulas (1) and (2) are easy to compute. They are somewhat cumbersome algebraically, however. The following approximation to the true rates, therefore, seems useful. If the proportion of the general population having cancer of the lung, X, is small relative to both the proportion of the control group smoking and not smoking, p_2 and $1-p_2$, the contribution of the term $X(p_1-p_2)$ to the denominator of formulas (1) and (2) is trivial and may be neglected. In that case the approximate rate of cancer of the lung among smokers becomes $\frac{p_1X}{p_2}$ and the corresponding rate for nonsmokers $\frac{(1-p_1)X}{1-p_2}$. Whenever p_1-p_2 is greater than zero, p_1/p_2 is greater than unity. We may conclude from the approximation, therefore, that whenever a greater proportion of the diseased than of the control group possess a characteristic, the incidence of the disease is always higher among those possessing the characteristic. This is the intuition on which the procedures used in such clinical studies

are based. Although it has frequently been questioned, it can now be easily seen to be correct.

It also follows from this analysis, however, that if one knows X, the prevalence of cancer of the lung in the general population, one can compute its prevalence among the smoking and nonsmoking population. Hospital or clinical records usually cannot furnish an estimate of X, however, since one seldom knows the size of the population exposed to risk from which the actual cases are drawn. Its value is frequently known, at least approximately, from other sources. Thus, we have estimated from Dorn's data (*2*) that the annual prevalence of cancer of the lung among all white males aged 40–49 is 15.5 per 100,000.[2] X consequently is equal to 0.155×10^{-3}. We may now construct a table showing the proportion of the population in each of the four categories from the data of Schrek *et al.*

	Having cancer of the lung	Not having cancer of the lung	Total
Smokers	0.119×10^{-3}	0.579910	0.580029
Nonsmokers	$.036\times10^{-3}$	.419935	.419971
Total	$.155\times10^{-3}$	.999845	1.000000

The proportion of smokers who have cancer of the lung using formulas (1) and (2) is thus 0.205×10^{-3} as contrasted with 0.086×10^{-3} for nonsmokers. The corresponding rates are 20.5 and 8.6 per 100,000 per year. These rates clearly provide a sounder basis for appraising the effect of cigarette smoking than does the knowledge that 77 percent of those with cancer of the lung and 58 percent without it smoke.

If one is interested only in knowing the relative amount by which the prevalence of the disease is augmented by the possession of the attribute, one may calculate this without knowledge of X, since the ratio of the two rates is $\frac{p_1}{p_2}\frac{(1-p_2)}{(1-p_1)}$ when X is small. One can thus conclude from the Schrek data alone that the prevalence of cancer of the lung among white males aged 40–49 is 2.4 times as high among those who smoke 10 or more cigarettes a day as among those who do not.

The more extensive, but age-standardized, data of Levin, Goldstein, and Gerhardt (*3*) on the same subject may be used to illustrate the same calculation. They show that 66.1 percent of all (presumably white) males at all age groups who had cancer of the lung smoked some cigarettes as compared with 44.1 percent smoking among the control group. Setting $.661=p_1$ and $.441=p_2$, we have $\frac{p_1}{p_2}\frac{(1-p_2)}{(1-p_1)}=2.5$. The prevalence of lung cancer, according to these data is 2.5 times as high among cigarette

[2] Dorn's published data show an annual prevalence rate in the period 1937–1939 of 29.7 per 100,000 for cancer of all respiratory organs among white and colored males, aged 40–49. In the North 52.1 percent of the respiratory cases in all age groups for both males and females was accounted for by lung cancer. The estimate of 15.5 (=29.7×0.521), is consequently somewhat rough.

smokers as among nonsmokers. (The agreement with the Schrek data is closer than would be expected in view of differences in the population covered, definitions used, and number of cases studied. The application of the present method to other studies of lung cancer and tobacco yields much more divergent results.)

The calculations may also be applied to multiple classifications such as the data on cancer of the cervix in Cardiff, Wales, recently published by Maliphant (*4*). In table 1, the first column gives the percent distribution of women who develop cancer of the cervix by marital status and number of children borne, while the second column shows the same distribution for all women. Women under 40 have been excluded. From other data given by Maliphant we have estimated that the incidence rate of cervical cancer for women over 40 in Cardiff was 79.7 per 100,000 (somewhat below the corresponding rate in this country.) This yields X and we accordingly have been able to calculate the incidence rates by marital status and number of children shown in the third column. The relation between cervical cancer and number of children born is obviously shown more clearly and usefully by the rates in the third column than by the relative frequencies in the first two.

TABLE 1.—*Distribution of women with and without cervical cancer by marital status and number of children*

	Women contracting cancer of the cervix, $100\,p_1$	All women, $100\,p_2$	Incidence rate per 100,000, $\frac{p_1 X}{p_2}$
Unmarried	1. 3	10. 5	9. 9
Married:			
No children	5. 0	13. 0	30. 7
1 or more children, total	93. 7	76. 5	97. 6
1 child	13. 3	15. 3	69. 3
2 children	18. 3	17. 0	85. 8
3 children	15. 0	13. 0	92. 0
4 children	11. 0	9. 6	91. 3
5 children	9. 2	6. 4	114. 6
6 or more	26. 9	15. 2	141. 6
Total	100. 0	100. 0	

TESTS OF SIGNIFICANCE ON THE COMPUTED RATE

Since most clinical studies are based on limited numbers of cases, it is of some importance to be able to estimate the limits of error of rates calculated according to this procedure. The approximate formula for the variance of a ratio sometimes used is inappropriate for this purpose, since it will sometimes show $\frac{p_2 X}{p_1}$ differing significantly from X when a test on the difference p_2-p_1 shows that it does not differ significantly from zero. To avoid this we employ a test of Fieller's (*5*). Thus, writing the computed prevalence rate as $\frac{p_1 X}{p_2} = r$ and denoting by

n_1 = the number of disease cases

n_2=the number of control cases

t_α=the value of t in the normal curve corresponding to the 100α-percent probability level

pq=the unbiased estimate of the unknown population value PQ

$$\left(=\frac{n_1p_1+n_2p_2}{n_1+n_2-1}\left[1-\frac{n_1p_1+n_2p_2}{n_1+p_2}\right]\right)$$

the upper and lower confidence limits for the 100α-percent probability level of the estimate r are given by

$$\frac{r\pm\frac{t_\alpha X}{p_2}\sqrt{\frac{pq}{n_1}\left[1+\frac{1}{n_2{p_2}^2}\left(n_1p_1^2-t_\alpha^2pq\right)\right]^{\frac{1}{2}}}}{1-\frac{{t_\alpha}^2pq}{n_2{p_2}^2}},$$

when X is considered free from sampling error. We may use the Schrek data to illustrate the use of this formula. Thus, letting n_1=35, n_2=171, setting t_α=2, and using p, X, and r as previously calculated, we compute the upper limit to the rate as 25.6 per 100,000 and the lower limit as 16.1 per 100,000. Since the value of X used, 15.5 per 100,000, falls outside these limits, we conclude that the rates for smokers and nonsmokers differ significantly at the 5-percent probability level. Whenever p_1 and p_2 differ significantly at the 100α-percent level, the limits computed in this fashion will not include X, and *vice versa*. Thus, if one simply wishes to test significance, it is sufficient to test the difference between p_1 and p_2. If one wishes to express error limits in the same units that the prevalence rate is expressed, however, one must use the formula given.[3]

PITFALLS

Our major purpose in preparing this note has been to show that any set of data that furnishes estimates of relative frequencies can be used to obtain estimates of rates. The procedure suggested, however, has assumed that the diseased and control groups used are representative of these same groups in the general population. If this assumption is not satisfied, then neither the rates, the relative frequencies, nor any other statistics calculated from the data will have applicability beyond the particular group studied.

We may illustrate the difficulties that can arise on this score with 2 examples. The first relates to Lane-Claypon's study of cancer of the breast (*6*). In this study a detailed questionnaire was filled in for 508

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[3] The procedure discussed in the text yields a two-sided test of significance; *i. e.*, it tests the hypothesis that the rate for smokers is significantly different from that for nonsmokers. It would be more realistic to use a one-sided test; *i. e.*, test the hypothesis that the rate for smokers is significantly *higher* than that for nonsmokers. To do this one uses the same formula but calculates only a lower limit, using a value of t_α appropriate to the one-sided test. Thus, for α=0.05, t_α=1.645.

In testing whether p_1 and p_2 are drawn from the same population it is appropriate to compute a pooled variance as has been done. When the results of such a test of significance suggest that p_1 and p_2 could not have been drawn from the same population, however, the use of a pooled variance to compute error limits is no longer correct. In fact, exact confidence limits can no longer be calculated for this case. The results yielded by the formula will nevertheless be sufficiently accurate for most practical purposes.

women with breast cancer and 509 control women, who were being treated by the cooperating hospitals for "some trouble, other than cancer." We reproduce in table 2 the percent distribution by number of children ever borne for each group. Only women having passed the menopause are included. We do not know X, the prevalence rate of breast cancer in the United Kingdom at the time the data were collected, and have therefore confined ourselves to computing relative prevalence.

TABLE 2.—*Distribution of women with and without breast cancer by marital status and number of children*

Characteristic	Cancer group, p_1	Control group, p_2	$\frac{p_1}{p_2}$	Relative prevalence
Unmarried	20. 91	16. 42	1. 273	100
Married:				
No children	14. 55	10. 45	1. 392	109
1 to 3 children	29. 09	24. 78	1. 174	92
4 to 6 children	21. 21	22. 39	. 947	74
7 or more	14. 24	25. 97	. 548	43
Total	100. 00	100. 00		

If the data are to be taken at their face value, one must conclude that lowered prevalence of breast cancer is associated with increasing numbers of children. Greenwood in an analysis of Lane-Claypon's data (*6*) in fact concludes, "we think then that an etiological factor of importance has now been fully demonstrated." At the very beginning of his analysis, however, he points out, without attaching any significance to it, that the control group had borne an average of about 25 percent more children than had all women in England and Wales with the same duration of marriage. This would appear to provide definite evidence for the unrepresentative character of the control group and to cast doubt on the adequacy of the evidence.

The basic difficulty in this example is the unrepresentative nature of the control group. Since there is always some doubt whether or not a control group selected from among hospital patients can provide an accurate estimate of the frequency of a characteristic in the population at large, the difficulty may be quite general. The possibility that the diseased group is not representative either, cannot be entirely disregarded, however. We reproduce in table 3 the distribution by age of 413 patients with adenocarcinoma of the breast admitted to the Ellis Fischel State Cancer Hospital in the years 1940–46 as given by Ackerman and Regato (*7*). For comparison we give the expected distribution on the basis of known incidence rates by age.

It is obvious from inspection that an excess number in the older age groups were encountered, and that to some extent the hospital was functioning as a home for the aged. An epidemiological investigation the results of which would be sensitive to the age distribution of the persons studied might consequently be adversely affected.

Any set of hospital or clinical data that is worth analyzing at all is

TABLE 3.—*Actual and expected distribution of breast cancer cases by age—Ellis Fischel State Cancer Hospital*

Age	Number of breast cancer cases [1]		(3) (4) × (5)	Incidence of breast cancer per 100,000 [2] (4)	Percent distribution Missouri population 1940 [3] (5)
	Reported (1)	Expected (2) (3) $\frac{\text{Tot. (1)}}{\text{Tot. (3)}}$			
Less than 30	3	7	1. 1	2. 2	0. 4825
30–34	10	13	1. 9	25. 1	. 0795
35–39	24	25	3. 7	50. 7	. 0774
40–44	26	41	6. 1	91. 1	. 0734
45–49	40	54	8. 0	122. 9	. 0674
50–54	51	51	7. 5	129. 0	. 0648
55–59	54	57	8. 4	169. 6	. 0580
60–64	54	53	7. 8	190. 4	. 0495
65–69	59	45	6. 6	193. 3	. 0410
70–74	48	34	5. 1	205. 5	. 0344
75 and over	44	33	4. 9	184. 5	. 0516
Total	413	413	61. 1	----------	1. 0000

[1] Chi square for difference = 24.5, $P<0.01$.
[2] As estimated by Dorn (*2*).
[3] U. S. Bureau of the Census, Population, vol. II, pt. 4, table 7.

worth analyzing properly. It is from this point of view that the technique proposed seems useful. The preceding two examples suggest, however, that the results of even the most carefully analyzed set of such data may be open to question, and that these doubts can be resolved only by methods of data collection that provide representative samples of diseased and nondiseased persons.

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A SEROLOGIC STUDY OF THE MICROSOME FRACTION OF NORMAL AND NEOPLASTIC MOUSE TISSUES [1]

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The term microsome was first used in 1943 by Claude (*1*) to describe one of the components isolated from mammalian cells by differential centrifugation. This fraction is considered to be cytoplasmic in origin (*2*). Recently the technique for isolating microsomes was modified by Hogeboom, Schneider, and Pallade (*3*) who recommended the use of 0.88 M sucrose instead of physiological saline as the suspending medium.

The serologic properties of microsomes isolated by the sucrose method from the tissues of normal and leukemic inbred mice have been reported by Dulaney *et al.* (*4*). By the use of the complement-fixation technique they demonstrated the presence of organ- and fraction-specific antigens as well as the Forssmann antigen in the microsome fraction. No qualitative difference was observed, however, when microsome fractions from the spleens of normal and leukemic mice were compared. These results are in agreement with the reports of earlier workers who used "heavy, sedimentable particles" (*5*, *6*, *7*) as antigens.

The following experiments represent a study of the serologic properties of the microsome fraction isolated by the sucrose technique from normal and neoplastic tissues of inbred mice.

MATERIALS AND METHODS

Preparation of Antigens

Strain C mice were used to provide liver and kidney tissue, *o*-aminoazotoluene-induced hepatomas (*8*) and spontaneous and transplanted mammary adenocarcinomas. Strain C3H mice provided liver, splenic tissue, and spontaneous and transplanted C3HBA mammary tumors (*9*). Splenic tissue and spontaneous and transplanted MT8 mammary tumors were derived from C3Hb mice. (C3Hb mice are C3H mice deprived of the milk agent by foster nursing.) Liver tissue was also obtained from strain C57 black mice. Inbred Family No. 2 guinea pigs were used as a source of liver tissue.[2]

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[2] We wish to express our thanks to Dr. H. B. Andervont who kindly supplied us with the hepatomas and the strain C spontaneous mouse mammary tumors; to Dr. W. E. Heston for the C3Hb spontaneous mammary tumors and the inbred guinea pigs; to Dr. L. W. Law for the C3Hb transplantable mammary tumor MT8; and to Dr. M. K. Barrett for the C3HBA transplantable mammary tumor.

The microsomal antigens used for immunizing rabbits were prepared exactly as described by Alfin-Slater *et al.* (*10*). This technique was slightly modified for the preparation of the antigens used in serologic studies. The modification consisted of allowing the microsome pellet to stand overnight at 5° C. before resuspension and final centrifugation. It was found that this method permitted the fractions to be prepared on one day and used in the serologic test the next day without affecting the complement-fixing properties of the microsomes.

When comparing the serologic properties of the microsomes with whole tissue brei and mitochondria, these fractions were set aside at the appropriate steps in the preparation of the microsomes.

Nitrogen determinations were done on all antigens using the Kock and McMeekin micro-Kjeldahl method (*11*) with Vanselow's modification (*12*) of Nessler's reagent. When used as antigens in serologic procedures, all fractions were made up in 0.85 percent saline to contain 0.03 mg. of protein nitrogen per cc.

Preparation of Antisera

Two or more 2–3 kilogram rabbits were used as a source of each antiserum. After a preliminary bleeding each rabbit was injected subcutaneously once a week for 3 weeks with a mixture of 2 cc. of freshly prepared antigen, 2 cc. of mineral oil, and 0.4 cc. of Arlacel A.[3] The amount of antigen given to each rabbit was based on protein-nitrogen content and represented a total of about 1.5 mg. of nitrogen per kilogram of body weight. Starting 1 week after the last injection, serum was collected from the ear vein twice a week for 2 weeks. All sera were separated from the clots and stored at −20° C. until used.

Serologic Method: The serologic techniques were carried out as described in a previous report (*13*). In most cases, however, tests using serial twofold dilutions of antisera were used as well as tests using the usual serial twofold dilutions of antigen.

Absorption studies were done on hepatoma microsome-fraction antisera and liver microsome-fraction antisera by absorbing with the heterologous microsome fraction at 5° C. overnight. Following absorption, the microsomes were removed by centrifugation at 20,000 × g for 4 hours. The supernatant was then used in the tests. Absorption studies were performed in the same way, using antisera prepared against the microsome fraction of tissues from mice with and without the milk agent as well as antisera to the mitochondrial and microsome fractions of the same tissue. Numerous complement-fixation tests were run on each of the various antigen-antibody combinations but only the results of typical experiments are presented in tabular form.

RESULTS

Complement-fixation titers on rabbit sera harvested before and after immunization showed that the microsome fraction is antigenic. The usual individual variation in the ability of rabbits to produce antibodies

[3] Kindly provided by the Atlas Powder Company, Wilmington, Del.

was noted, but in general, the microsome fractions were good antigens.

Studies of the effect of temperature and method of storage upon the complement-fixing ability of the microsome fraction indicated that both these factors were important. It was found that there was a decrease in the complement-fixing ability of the microsome fraction when stored overnight at 5° C. suspended in sucrose or when frozen either in pellet or suspended form. However, when stored overnight at 5° C. in pellet form the complement-fixing ability was not affected. Freshly prepared microsomes were used in serologic tests to compare the fractions stored in these various ways.

The greater reactivity of a typical microsome fraction with its homologous antisera is demonstrated in table 1. The microsome fraction reacted more strongly with the microsome-fraction antisera than did the mitochondrial fraction isolated from the same tissue. Absorption of the microsome antisera with mitochondria and the absorption of the mitochondria antisera with microsomes did not increase the specificity of the reaction.

A demonstration of species specificity of microsomes is shown in table 2, where microsomes of both guinea-pig liver and mouse liver reacted more strongly with their homologous antisera.

No strain specificity was demonstrated by the microsome fraction of liver tissue from strains C, C57 black, and C3H. As shown in table 3, the microsomes reacted equally well with the microsome-fraction antisera from the homologous organ of these strains. C and C3H mouse mammary tumor microsome antisera also failed to differentiate strains.

The microsome fractions of strain C liver and kidney tissues were found to contain antigens that reacted more strongly with their homologous antisera. This is illustrated in table 4.

The mammary tumor milk agent does not appear to be concentrated in the microsome fraction. The microsome fractions of mammary tumor from strains C3H and C3Hb react equally well with the microsome-fraction antisera from mammary tumors of either strain. The same result is obtained when the C3H and C3Hb mammary tumor microsome-fraction antiserum is tested against C3H and C3Hb splenic microsomes. An antiserum to the ultracentrifuge pellet of C3H or C3Hb mammary tumors, when tested against microsome fractions of C3H and C3Hb mammary tumors or spleens, also failed to show the presence of the milk agent.

Cross-absorption tests with these sera also failed to elicit any serologic difference between the C3H and C3Hb microsomes.

No qualitative difference was found between the microsome fractions of strain C liver and hepatoma. The microsomes of both tissues reacted more strongly with the hepatoma microsome antisera (table 5). Absorption of both antisera with heterologous antigen did not increase the specificity of the reaction (table 6). After absorption, both the liver and hepatoma microsome-fraction antisera reacted equally well with either antigen although in lower titer than the unabsorbed antisera.

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TABLE 1.—*Complement-fixation reaction of microsome and mitochondrial fractions from a C3H mammary tumor with a microsome antiserum*

Antisera to—	Antigen	Complement fixed by antigen dilution					
		1:2	1:4	1:8	1:16	1:32	1:64
Microsome fraction of C3H mammary tumor.	C3H mammary tumor microsome fraction.	++++	++++	++++	++++	++++	0
	C3H mammary tumor mitochondrial fraction.	++++	++++	++++	0	0	0

TABLE 2.—*Complement-fixation reactions of microsome fractions from mouse and guinea-pig liver tissues with their antisera*

Antisera to microsome fraction of—	Source of microsome fraction used as antigen	Complement fixed by antigen dilution					
		1:2	1:4	1:8	1:16	1:32	1:64
Family No. 2 guinea-pig liver tissue.	Guinea-pig liver	++++	++++	++++	++++	++++	0
	Mouse liver	++++	++	0	0	0	0
Strain C mouse liver tissue	Guinea-pig liver	++++	0	0	0	0	0
	Mouse liver	++++	++++	++++	++++	++	0

TABLE 3.—*Complement-fixation reaction of microsome fractions from strains C and C3H mouse liver with their antisera*

Antisera to microsome fraction of—	Source of microsome fraction used as antigen	Complement fixed by antigen dilution					
		1:2	1:4	1:8	1:16	1:32	1:64
Strain C mouse liver	Strain C mouse liver	++++	++++	++++	++++	0	0
	Strain C57 black mouse liver	++++	++++	++++	++++	0	0
	Strain C3H mouse liver	++++	++++	++++	++++	0	0
Strain C3H mouse liver	Strain C mouse liver	++++	++++	++++	0	0	0
	Strain C57 black mouse liver	++++	++++	++++	0	0	0
	Strain C3H mouse liver	++++	++++	++++	0	0	0

TABLE 4.—*Complement-fixation reactions of microsome fractions from strain C mouse liver and kidney with their antisera*

Antisera to microsome fraction of—	Source of microsome fraction used as antigen	Complement fixed by antigen dilution					
		1:2	1:4	1:8	1:16	1:32	1:64
Liver	C mouse liver	++++	++++	++++	++++	0	0
	C mouse kidney	++++	0	0	0	0	0
Kidney	C mouse liver	++++	0	0	0	0	0
	C mouse kidney	++++	++++	++++	++++	++++	0

TABLE 5.—*Complement-fixation reactions of microsome fractions from strain C mouse liver and hepatoma with their antisera*

Antisera to microsome fraction of—	Source of microsome fraction used as antigen	Complement fixed by antigen dilution						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
C mouse liver	C mouse liver	++++	++++	++++	++++	0	0	0
	C mouse hepatoma	++++	++++	++++	++++	++++	++++	0
C mouse hepatoma	C mouse liver	++++	++++	++++	++++	0	0	0
	C mouse hepatoma	++++	++++	++++	++++	++++	++++	0

TABLE 6.—*Complement-fixation reaction of microsome fractions from strain C mouse liver and hepatoma with their antisera after the antisera were absorbed with the fraction from the heterologous tissue*

Antisera to microsome fraction of—	Source of microsome fraction used as antigen	Complement fixed by antigen dilution						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
C mouse liver	C mouse liver	++++	0	0	0	0	0	0
	C mouse hepatoma	++++	0	0	0	0	0	0
C mouse hepatoma	C mouse liver	++++	0	0	0	0	0	0
	C mouse hepatoma	++++	0	0	0	0	0	0

TABLE 7.—*Complement-fixation reactions of C mouse mammary tumor and hepatoma and C3H mouse mammary tumor with their antisera*

Antisera to microsome fraction of—	Source of microsome fraction used as antigen	Complement fixed by antigen dilutions					
		1:2	1:4	1:8	1:16	1:32	1:64
C3H mammary tumor	C3H mammary tumor	++++	++++	++++	++++	++++	0
	C mammary tumor	++++	++++	++++	++++	++++	0
C3H mammary tumor	C3H mammary tumor	++++	++++	++++	++++	++++	0
	C Hepatoma	++++	++++	++++	0	0	0
C mammary tumor	C mammary tumor	++++	++++	++++	++++	0	0
	C3H mammary tumor	++++	++++	++++	++++	0	0
C mammary tumor	C mammary tumor	++++	++++	++++	++	0	0
	C hepatoma	0	0	0	0	0	0
C hepatoma	C hepatoma	++++	++++	++++	++++	++++	++++
	C3H mammary tumor	++++	++++	++++	++++	0	0
C hepatoma	C hepatoma	++++	++++	++++	++++	++++	0
	C mammary tumor	++++	++++	0	0	0	0

When a comparison was made between the microsome fractions of two different tumors, it was found that each tumor reacted more strongly with its homologous antisera. As seen in table 7, the C hepatoma microsome fraction reacted more strongly with C hepatoma microsome antisera and the C mammary tumor microsome fraction reacted more strongly with its antisera. When C hepatoma and C3H mammary tumor microsome fractions were compared they also reacted more strongly with their homologous antisera.

No difference could be detected, however, between the microsome fractions of C3H and C mammary tumors.

DISCUSSION

The results of this study indicate that microsome fractions from normal and neoplastic tissues of inbred mice and guinea pigs react more strongly in complement-fixation tests with antisera to the microsome fraction from the homologous organ and species.

It is evident that the microsome and mitochondria fractions react more strongly with their homologous antisera (table 1). It cannot be determined from these results, however, whether this greater reactivity is due to a quantitative difference in the antigens of the two fractions or to the presence of different antigens in the two fractions.

If the factors involved in histocompatability are measurable by complement fixation they would not appear to be concentrated in the microsome fraction. This is illustrated in table 3, where strain-specific antigens could not be detected in the microsome fraction of the tissues of strains C, C57 black, and C3H mice.

It has been reported (*14*, *15*, *16*), that the mammary tumor milk agent can be detected by serologic tests. The inability to detect the presence of the agent in microsomes and mitochondria (*13*) may be a result of the isolation technique used. At this point it seems safe to say that the milk agent, if detectable by the complement-fixation test used, is not concentrated in mitochondria or microsomes isolated by the sucrose method.

There are several possible explanations for the greater reactivity of the hepatoma microsome fraction as compared with the liver microsome fraction; however, on the basis of our experiments, no conclusive interpretation can be given. Similar relationships between normal and neoplastic-tissue fractions have previously been noted in mitochondrial (*4*, *13*) and microsome (*4*) fractions.

The specificity observed in comparing mammary tumor microsome fractions with hepatoma microsome fractions may be an expression of organ specificity, since organ-specific antigens were shown to be present in microsome fractions (table 4).

It is apparent from this study as well as that of Dulaney *et al.* (*4*) that although the microsome fraction shows greater reactivity with its homologous antisera when compared with the mitochondrial fraction from the same tissue, the general serologic properties of the two fractions are the

same. That is, both are antigenic, both react more strongly with antisera against a homologous organ and species, and neither fraction shows tumor-specific or strain-specific antigens.

SUMMARY

Microsome fractions isolated by the sucrose technique from normal and neoplastic tissues of inbred mice were studied serologically using the complement-fixation reaction. The microsome fractions contain antigens that react more strongly with antisera prepared against the homologous organ and species. In a serologic comparison of microsomes from normal liver and hepatoma tissue, quantitative but not qualitative differences were observed. Serologic comparison of microsome fractions from mammary tumor and hepatoma tissue revealed qualitative differences that were interpreted as expressions of organ specificity. The mammary tumor milk agent was not detectable in the microsome fraction.

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THE ACTION OF TISSUE EXTRACTS ON CELL DIVISION [1] [2]

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In our search for antimitotic substances that would be effective without being too toxic, we have proceeded on the basis of the theory that all living material contains substances that favor protoplasmic clotting and those that tend to prevent such clotting. The clotting of protoplasm, similar basically to the clotting of vertebrate blood, is an essential prerequisite for the formation of the mitotic spindle. In other words, mitotic gelation necessarily precedes spindle formation. If we could treat the protoplasm so that it is kept fluid, then we would expect to be able to prevent the mitotic gelation, and we could thus stop the division of the cell. The basis for this point of view has been discussed in earlier publications (*1*, *2*, also references cited there).

If protoplasm in its colloidal behavior is so much like blood, then it might be thought that heparin would prevent protoplasmic clotting in general, and mitotic gelation in particular. This indeed it does do (*3*). Likewise the bacterial polysaccharide of Shear, a substance that has a marked, irreparable, damaging effect on tumors in mice, with but minimal injury to the normal tissues of these animals, acts on protoplasm like heparin, only more strongly (*4*, *5*). Actually, Shear's polysaccharide appears, in some respects at least, to be similar to heparin, and it can be shown to have an anticlotting effect on human blood. Far weaker than heparin in its effect on blood, it is nevertheless much more potent than heparin in its action on protoplasm. From a practical standpoint, perhaps the ideal antimitotic substance would be one that had relatively little effect on blood and very much effect on protoplasm. Also, as is obvious to all students of cancer, it would be advantageous if we knew of substances that were in some degree specific in their action on certain types of cells rather than others.

Clearly, there is a need to study as many antimitotic substances as possible. Starting with the thesis that protoplasm, like blood, is poised in a delicate balance between the factors that tend to cause clotting and those that tend to prevent it, we began a search for naturally occurring

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substances that would keep the protoplasm fluid and inhibit protoplasmic clotting.

Because of the fact that heparin is a metachromatic substance and stains red with toluidine blue, it is to some extent possible to discover its presence in various types of cells. One of our group, Mr. John W. Kelly, has been making a survey to indicate the presence of heparin or heparin-like substances in various types of cells and in different parts of these cells. The occurrence of heparin or heparin-like substances is certainly widespread if not universal in living systems. It is doubtless more widespread than cytochemical tests based on metachromatic reactions will show. For when heparin is completely bound to protein, it loses its metachromasy. This is the reason, probably, that heparin cannot be shown to be present in vertebrate muscle by the toluidine blue test in spite of the fact that muscle is especially rich in the substance (*6*).

Guided somewhat by the metachromatic studies, we began a search for the antimitotic substances we believed to be present in various types of living material. Our first attempts to obtain anticlotting and antimitotic substances were not very successful. In these attempts we were influenced by the knowledge that the substances in cells that promote the clotting of blood are soluble in alcohol. This had been shown over and over again by Alexander Schmidt and his pupils (*7*, *8*). Similarly, the work in our laboratory had shown that protoplasmic clotting was induced by alcoholic extracts of injured tissues (*9*, *10*). On the basis of this information, we decided to make extracts from the alcohol-insoluble fraction of living materials. However, we had little or no success with this type of extract. We decided therefore to attempt another approach.

One of the cellular materials richest in heparin or heparin-like substances is the jelly of sea-urchin eggs [for discussion, see (*11*)]. Sea-urchin-egg jelly, as has long been known, is soluble in acidified sea water, and we began our studies first by extracting marine eggs with acidified sea water, for we knew that the "heparin" in the jelly of the eggs would come out into the extracts. Later we extended our studies to include other types of living material.

MATERIALS AND METHODS

In most of our work, eggs of the annelid worm *Chaetopterus pergamentaceus* were used as test objects. In a few experiments, we used eggs of the sea urchin *Arbacia punctulata*. In all cases, the eggs were kept in a constant temperature bath maintained at 21° C.

When acid is added to sea water, the bicarbonates break down and carbon dioxide is released. This gradually escapes so that the solution has a changing pH. In order to obtain acid sea water of reasonably constant pH, we added HCl to the sea water in sufficient quantity to break down the bicarbonates. Then the acidified sea water was placed in shallow dishes, usually over night, so that the carbon dioxide could diffuse away. After the carbon dioxide had escaped, the acidified sea water, no longer buffered with bicarbonates, could readily be brought to any desired

hydrogen-ion concentration by the addition of dilute HCl or NaOH. In preparing extracts, we used approximately equal volumes of the living material and of the acidified sea water. After a short time interval—usually 30 minutes—the extract was centrifuged to remove suspended undissolved material. It was then diluted with ordinary sea water. This in every case brought the pH above 7, usually well above 7. Control experiments with solutions of varying pH showed that it was safe to use solutions of pH 6.8 or above.

In measurements of protoplasmic viscosity, an Emerson hand centrifuge was used. For details about the technique of viscosity measurements, see (*11*).

RESULTS

In our first experiment, we made an acid sea-water extract of the jelly of Chaetopterus eggs. In order to obtain Chaetopterus eggs from the worm, the posterior segments of the animal are cut into small pieces; the eggs stream out into the sea water; they are then strained through cheesecloth. The cloth through which the eggs have been strained is covered with slime, which presumably consists largely of the jelly that has surrounded the eggs. We extracted this slime and obtained solutions that had some effect in retarding the cleavage of fertilized eggs. More active extracts were obtained by grinding up the posterior segments of the worm in acid sea water (pH 4.9). One such extract, when diluted with an equal volume of sea water (the pH of the diluted extract was 7.2), caused a marked delay in the cleavage of Chaetopterus eggs. Other experiments gave similar results.

We also made acid sea-water extracts of ovaries of the sea urchin Arbacia and the sand dollar Echinarachnius, and these extracts likewise caused a retardation and to some extent an inhibition of cleavage in the eggs of Chaetopterus. An extract of the ovary of the clam *Spisula solidissima* (commonly but incorrectly called *Mactra solidissima* by embryologists) caused a marked retardation of cleavage, and an extract of whole clams (*Venus mercenaria*) likewise had some retarding effect.

After these early experiments, we tried an extract of ovaries of the starfish. The results were so striking that we abandoned all other types of material and in the time that was remaining during the summer of 1950, we concentrated our attention on the starfish ovaries. In making extracts from starfish ovaries, the following procedure was employed. The ovaries were allowed to remain in contact with the acidified sea water for 30 minutes (or in a few cases for 45 minutes); then the preparation was centrifuged and the supernatant solution was used. The results of a series of tests are shown in table 1. In this table, the pH of the acid sea-water extract is given in the third column; in the fourth column the dilution is given in terms of the volume of acid sea-water extract (the first figure) and volume of ordinary sea water added (the second figure). The pH of the resultant mixture is given in the fifth column. The "polar body count" as given in the sixth column does not represent the actual percentage of eggs with polar bodies. If eggs, 100 percent of which have

polar bodies, are examined under a microscope, only about one egg in three shows its polar body if the eggs are allowed to remain quietly on the slide. If the eggs are moved about, then of course it is possible to see a polar body attached to each egg. In order to convert polar-body counts as given in the table into actual percentages of eggs with polar bodies, it is only necessary to multiply the figure given by three. In some of the experiments, because of the treatment given the eggs, the polar bodies were sometimes larger than normal. This larger size made them somewhat more visible, and they showed in perhaps a little more than a third of the eggs counted. This, however, constitutes only a minor source of error.

In the experimental eggs, the cleavage count may vary with time. This is due to the fact that there may be a delay in cleavage, so that for a time, at least, the number of divided eggs keeps increasing. However, when the action of the extract is strong, none of the exposed eggs divides; most of them disintegrate.

In all the experiments recorded in table 1, the eggs were placed in the solutions containing starfish extract for 10 minutes before spermatozoa were added. Controls of eggs in normal sea water were inseminated at the same time. These always gave percentages of cleavage that approximated 100 percent.

TABLE 1.—*Effect of starfish extract on division of Chaetopterus eggs (eggs exposed 10 minutes before fertilization)*

Experiment No.	Extract	pH during extraction	Dilution	Final pH	Polar-body count	Percent cleavage
1	S1	4.5	1–1	7.32	0	0
2	S1	4.5	1–3	7.75	0	0
3	S1	4.5	1–9	7.80	40	0
4	S1	4.5	1–24	7.92	10	76
5	S2	4.5	1–1	7.68	32	63
6	S2	4.5	1–9	7.86	32	99
7	S3	4.5	1–1	7.68	29	2
8	S4	4.5	1–1	7.70	0	0
9	S5	4.5	1–1	7.78	32	21
10	S3	4.5	1–1	7.18	16	0
11	S4	4.5	1–1	7.20	0	0
12	S5	4.5	1–1	7.22	12	58
13	S7	5.5	1–9	7.82	31	1
14	S7	5.5	1–19	7.85	34	96
15	S7	5.5	1–4	7.72	9	0

Table 1 shows clearly that extracts of starfish ovaries have a powerful effect in preventing cell division. This effect is not due to the presence of eggs in the ovaries. As a matter of fact, the ovaries from which the most active extracts were obtained were those that contained only few eggs, whereas the less potent extracts came from ovaries heavily laden with eggs. Thus, S1, S4, and S15 were extracts made from ovaries with few eggs, whereas S2, S3, S5, and S14 came from ovaries containing many eggs. When eggs themselves were extracted with acid sea water, they ave only a weak extract.

When the extract is potent, the eggs treated with it not only fail to divide, but often disintegrate. The reason for this will become apparent later; it is almost certainly due to a liquefaction and consequent weakening of the outer region of the egg.

In experiments in which eggs are exposed to substances before fertilization, an effect on cell division may be due to an action on fertilization or to an action on mitosis. It is clear, however, that in at least some of the experiments shown in table 1, the action of the extract must have been on the cleavage mitosis. Thus, in experiments 3, 7, 9, and 13, the polar-body count indicates that practically all of the eggs were fertilized and went through at least one maturation division, and yet only a small percentage of these eggs went on to cleave.

In another series of experiments, the eggs were exposed to the extracts only after fertilization had already occurred. The results are shown in table 2. In this series of experiments, the eggs were in every case exposed to the extracts 2 minutes after they had been inseminated.

TABLE 2.—*Effect of starfish extract on division of Chaetopterus eggs (eggs exposed 2 minutes after fertilization)*

Experiment No.	Extract	pH during extraction	Dilution	Final pH	Polar-body count	Percent cleavage
1	S4	4. 5	1–1	7. 70	0	0
2	S7	5. 5	1–4	7. 65	----------	17
3	S7	5. 5	1–9	7. 82	34	26
4	S7	5. 5	1–4	7. 72	32	1
5	S7	5. 5	1–19	7. 85	4	70
6	S8	5. 5	1–1	7. 15	28	35
7	S13	5. 28	1–1	7. 62	0	0
8	S13	5. 28	1–4	7. 68	0	0
9	S13	5. 28	1–9	7. 81	1	0
10	S14	5. 3	1–4	7. 78	----------	82
11	S15	5. 28	1–4	7. 79	----------	0
12	S22	5. 55	1–3	7. 53	----------	0

The results shown in table 2 are clear cut. In only one experiment (No. 10) was the effect on cell division not very pronounced. In this case the extract was too dilute to be effective; it was a rather weak extract for it came from an ovary rich in eggs. As previously noted, such ovaries never yielded strong extracts.

In the experiments recorded in tables 1 and 2 we used simple watery extracts of starfish ovary. In addition to these experiments, we made numerous other studies in which we attempted to separate the alcohol-soluble substances of the extracts from the alcohol-insoluble substances. These experiments, which will be referred to again later, gave us additional evidence that in the starfish ovary there was a substance that had a powerful antimitotic action.

The antimitotic potency of this substance could be related to its effect on the colloidal properties of the egg protoplasm. It has a very marked liquefying action on the protoplasm of the Chaetopterus egg and it prevents the appearance of the mitotic gelation. In our earlier studies we followed the course of viscosity change in the protoplasm of the Chaetopterus egg

from fertilization to cleavage (*2*). Using an Emerson hand centrifuge we found that when eggs were centrifuged at a centrifugal force 2,385 times gravity (one turn of the handle in 2 seconds), for most of the period between fertilization and cleavage, only 7 seconds of centrifugal treatment were necessary to displace light and heavy granules of the protoplasm to opposite ends of the egg. This represents an arbitrary viscosity value of 7. However, there is a sharp increase in protoplasmic viscosity that begins at about 26 minutes after fertilization (at 21° C.) and comes to a peak about 30 minutes after fertilization. For 10 minutes, from 30 minutes after fertilization until 40 minutes after fertilization, the viscosity remains high. It is approximately twice as high as it is before the rise in viscosity occurs. This constitutes the mitotic gelation, which involves an increase in viscosity to a value of 14 from a value of 7. But when the eggs are exposed to extracts of starfish ovary, if the extract is sufficiently concentrated, the protoplasm never undergoes a mitotic gelation and the viscosity drops until it may reach a value as low as 4. Numerous experiments were performed, and consistent results were always obtained. Never in our experience have we seen so strong an effect on the protoplasmic viscosity. The eggs become so fluid that they tend to disrupt, so that after a time viscosity measurements are no longer significant.

In order to illustrate the effect of the starfish-ovary extract on the protoplasmic viscosity, we shall choose an experiment in which the potency of the extract was not too great. After first trying a 1:1 dilution of the S7 extract of starfish ovary, we found this so strong that the eggs exposed to it tended to break up. Accordingly, we then made a 1:4 dilution of this extract. Two minutes after insemination, Chaetopterus eggs were placed in this solution, and samples of these eggs were centrifuged at 5-minute intervals. In each case the centrifugal treatment lasted for 6 seconds, and we then determined whether or not the viscosity value at a given time was higher than 6. In other words, we observed whether the 6-second treatment caused the granules of the egg cell to shift to such an extent that they gave the appearance of zones. The results of these tests are shown in table 3. Because of the fact that the extract in the

TABLE 3.—*Viscosity of the protoplasm of fertilized Chaetopterus eggs exposed to starfish extract*

Minutes after fertilization	Viscosity
5	6 or less.
10	Do.
15	Do.
20	Do.
25	Do.
30	38 out of 50 eggs have a viscosity of 6 or less.
35	39 out of 50 eggs have a viscosity of 6 or less.
40	37 out of 50 eggs have a viscosity of 6 or less.
45	49 out of 50 eggs have a viscosity of 6 or less.
50	6 or less.
At 54 minutes, 54 percent of the normal control eggs cleaved.	

dilution we used was not very potent, some of the eggs showed a viscosity increase that began at about 30 minutes after insemination and lasted for about 10 minutes. In a variable group of eggs it is very difficult after a given centrifugal treatment to determine exactly how many eggs have responded to the centrifugal treatment and how many have not. It is necessary to roll the eggs to observe them properly. We made counts as best we could, in each case counting 50 eggs for each test. As the table shows, in the 3 tests made between 30 and 40 minutes after insemination, we found that on the average 24 percent of the eggs showed some indication of viscosity increase during this time period when normally mitotic gelation occurs. How great the increase in viscosity was we had no way of knowing; but no doubt some of the eggs became more viscous than others. Cleavage counts made some time later showed that 17 percent of the eggs divided, so that we can assume most of the eggs that showed some degree of mitotic gelation went on to divide.

The starfish extracts not only caused a liquefaction of the protoplasm in the interior of the cell, but also caused a liquefaction of the protoplasm in the cortex. Ordinarily, when Chaetopterus eggs are centrifuged, there is no movement of the cortical granules, and these persist as a layer surrounding the more fluid interior of the egg cell. Because of this cortical layer, the hyaline zone in centrifuged eggs does not appear perfectly transparent. In our centrifuge studies we noticed that eggs exposed to starfish extracts typically showed a relatively clear hyaline zone. We were therefore led to a study of the viscosity of the cortical protoplasm.

In the first place, we found that it was possible to make measurements of the viscosity of the cortex of Chaetopterus eggs. Up until the present time, no proper measurements have been made of the viscosity of the cortex of metazoan cells, and it has been necessary to base all our information about the colloidal properties of the cell cortex on information obtained from the ameba (for discussion of this information, see *13*). It is true that Brown (*14*), and also Marsland (*15*) have published data on the viscosity of the cortex of sea-urchin (Arbacia) eggs, but the values that were obtained by both Brown and Marsland were based on the speed with which the pigment granules of the eggs were moved by centrifugal force. This was on the assumption that all the pigment granules of fertilized eggs were in the cortex, an assumption quite contrary to fact. For further discussion, see (*16*).

The important point is that in the Chaetopterus egg it is possible to make determinations of the amount of force that is necessary to make granules move out of the cortex. The force necessary to accomplish this movement is a measure of the stiffness of the cortical gel. When unfertilized Chaetopterus eggs are placed in an Emerson hand centrifuge and the handle of the centrifuge is then turned at the rate of one turn per second, the granules are torn loose from the cortex and go to the heavy pole of the egg. Usually in our studies we centrifuged for a full minute and then examined the eggs under the high power of the microscope. After this treatment, all or practically all of the granules have disappeared

from the cortex. One turn of the handle per second develops a centrifugal force of 9,300 times gravity. This then is a force sufficient to move the granules from the cortex. Following fertilization, the rigidity of the cortex decreases somewhat, then for a time it is high; 15 minutes before cleavage, and up to and during the actual division of the cell, the rigidity of the cortex is at its lowest. The exact time course of these changes and their approximate magnitude will be shown in another paper (*16*). Suffice to say at this point that the opinion of Marsland (*15*)—that the rigidity of the cortex is at its highest during the division of the cell—is certainly wrong for the Chaetopterus egg.

Our interest, of course, is in the effect of the starfish extract on the viscosity or rigidity of the cortex. As in the case of gels generally, the viscosity of the cortex is doubtless dependent on the shearing force exerted upon it. We are interested in determining the amount of centrifugal force necessary to tear granules loose from the cortical layer. In normal (unfertilized) eggs, one turn of the handle of the Emerson centrifuge per second develops a force strong enough to displace the cortical granules, but when the handle is turned 50 times in 60 seconds, the centrifugal force developed is not sufficient to move the granules. On the other hand, eggs exposed to starfish extract for 35 minutes showed a movement of cortical granules when the handle of the centrifuge was turned only 12 times in 60 seconds. This represents a centrifugal force only $\frac{1}{25}$th of the force developed when the handle is turned 60 times in 60 seconds. Thus, the rigidity of the cortex is decreased about 25 times by the action of the starfish extract. The effect of the extract is apparently rapid, but the exact length of time necessary to produce liquefaction was not determined. Apparently, heparin may also cause a liquefaction of the cortex; at least some types of heparin do. We found that two preparations of heparin made by Upjohn and Co., Kalamazoo, Mich., caused a fourfold decrease in the rigidity of the cortex. One of these heparin preparations came from pig lung, the other from dog liver. Only one experiment was performed, and in this experiment the eggs were exposed to a 0.1-percent solution of each heparin preparation for 5 hours. The heparin preparations were kindly given to us by Mr. Norman G. Anderson of Duke University, who obtained them from the Upjohn Company.

When sea-urchin (Arbacia) eggs are exposed to the starfish extract, they behave much as the Chaetopterus eggs do. We did only 2 experiments. In both, we used an extract prepared by exposing starfish ovaries to a solution composed of 3 parts of acidified sea water plus 1 part of distilled water. The extract was evaporated to dryness and then ½ gm. of the dry material was dissolved in 15 ml. of a mixture of sea water and distilled water. In one experiment the resultant solution had a freezing-point lowering of 1.88° C., in the second experiment the freezing-point lowering of the solution was 1.90° C. Thus both solutions were essentially isotonic with sea water. They were both mixed with sea water in the ratio of 1 part of extract solution to 2 parts of sea water. Sea-urchin (Arbacia) eggs were fertilized, and 2 minutes later some of them were placed in the

diluted starfish-extract solutions. Then centrifuge tests, both of the treated eggs and the controls, were made at frequent intervals. The control eggs showed the usual typical mitotic gelation, whereas the treated eggs, although they did show a slight increase in protoplasmic viscosity at the time the gelation was occurring in the controls, were always far more fluid. Thus the main mass of the protoplasm is kept fluid as a result of the exposure to the starfish extract.

The starfish extract also has a marked effect on the cortex of the sea-urchin eggs. When normal eggs, either before or after fertilization, are subjected to a centrifugal force of 9,300 times gravity, the cortical granules always remain in place. But in the eggs treated with starfish extract, the granules speedily moved out of the cortex when the eggs were centrifuged with a force 9,300 times gravity.

In these eggs treated with starfish extract and then centrifuged, a rather remarkable fact was observed. Normally in the sea-urchin egg, at least in the egg of Arbacia, the nucleus is lighter than the rest of the protoplasm and on centrifugation it moves toward the light pole and comes to rest immediately under the layer of fat or oil that accumulates at this pole. But in the treated eggs, as the time after fertilization (and exposure to the starfish extract) increased, the nuclei became heavier than the clear substance of the protoplasm, and on centrifugation they tended to move toward the heavy pole of the egg, usually coming to rest just above the layers of granules. Often the male and female pronuclei could be seen at the boundary of the hyaline zone and the granular layers. In these experiments with Arbacia eggs, the starfish extract stopped cell division; in one experiment the stoppage was complete, in the other only one egg out of a hundred counted showed division. The effect was reversible, for when the eggs were returned to sea water after an hour's exposure to starfish extract they tended to divide and develop normally. At least this was true in one experiment. In this case, 78 percent of the eggs divided on return to sea water. In the second experiment, 27 percent of the eggs divided on return to sea water.

Because starfish are so common along the Atlantic coast, the substance we extract from the ovaries can readily be obtained in quantity, and it can be obtained at almost any season of the year. We planned therefore to collect the substance during the winter months. However, when the gonads of starfish are small, there is a possibility that in hasty dissections of large numbers of animals we might at times include some testes in the material we dissected out of the starfish. Accordingly, we were interested in knowing whether the addition of testes to ovarian tissue would perhaps neutralize or otherwise adversely affect the isolation of the potent substance we were interested in. Experimentation soon showed that the testis itself contained the potent substance, and that moreover when extracts of testis and ovary were combined, there was no weakening of the activity of the extracts. We took steps therefore to assure for ourselves a supply of starfish during the winter. Along the New Jersey coast, starfish are not very numerous, at least in the part

of the coast near our laboratory in Philadelphia. We were fortunate to obtain the aid of F. Mansfield and Sons Co., New Haven, Conn., and of its treasurer, Mr. J. R. Nelson. The Mansfield Co. has been kind enough to send us numerous shipments of live starfish, and in view of the distance between New Haven and Philadelphia, these starfish have arrived in rather good condition. At any rate, we have used them to obtain extracts of ovarian material.

At the present time we are proceeding with studies of the chemical nature of the active substance in the extracts. Our work at Woods Hole had already indicated that the extraction of the ovaries had to be made in acidified sea water; in alkaline sea water no active substance comes out. The exact pH of the acid sea water does not seem to matter much. Likewise, as far as our present information goes, the temperature at which the extraction is made does not seem to be important. Effective extracts were obtained from ovaries extracted at 0° C., and those extracted at 50–55° C. The extracts can be boiled and the potency remains, although perhaps there is some loss. Indeed, the extracts can be boiled to dryness and the dried material is still effective.

At Woods Hole we did numerous experiments to determine whether the active substance we obtained from the starfish ovaries was alcohol soluble or not. Our first experiments indicated that the substance we were concerned with was relatively insoluble in alcohol. In these experiments, most of the activity appeared in the alcohol-insoluble fractions. However, later in the summer, relatively more of the activity appeared in the alcohol-soluble fractions. This puzzled us for a time, but there is a simple and reasonable explanation: polysaccharides, like heparin, tend to combine with lipids, and when they are so combined, they show a solubility in fat solvents. Indeed, when heparin was first discovered it was thought to be a lipid (*17, 18*). If the active substance of our starfish extracts is really a heparin-like compound, then it would tend to combine with lipids, and depending on the nature and degree of such combination, its solubility in fat solvents would vary. Actually, there is direct evidence that the starfish ovaries do contain heparin or heparin-like substances; this is in addition to the histochemical evidence cited previously. From the ovaries, Mr. Lyell Thomas in this laboratory has extracted with fat solvents a material that on further purification has an effect like heparin in preventing the coagulation of (chicken) blood. The anticlotting action of this substance is neutralized by protamine, and this constitutes additional evidence that it is heparin.

DISCUSSION

There are many types of substances that suppress cell division. Some of them are general protoplasmic poisons and as such they are of no particular interest, either for the theoretical understanding of the mechanism of cell division or for the practical question of cancer therapy. The fact that heparin and heparin-like substances are present in a wide range of living materials is an indication that they may play a part in the series

of colloidal changes that occur during the normal division of a cell. Moreover, there is a possibility that the curative effect of radiation on cancer may in part be due to the excess of heparin that appears in the blood after radiation treatment (*cf. 3*). Likewise, within the cell, the effect of roentgen rays may in some manner involve the action of the rays on heparin-protein combinations. Hence, both from a theoretical and a practical standpoint, it is important to study the antimitotic effect of heparin or heparin-like compounds, especially if it is true, as we believe, that essentially all cells contain such compounds.

Although in our extraction procedure we used a method that can be assumed to extract heparin-protein compounds, we have no certain knowledge that the extracts we obtained owe their activity to heparin or any compound similar to it. We do know that the ovary of the starfish is rich in heparin or some substance that behaves like it. Perhaps the ovaries of many organisms are rich in heparin-like antimitotic substances. Such substances might have a functional significance in preventing the division of the egg cells before it is time for them to divide.

In our search for substances that act like heparin on protoplasm, we are of course interested in finding substances that can penetrate the cell without too much difficulty. Ordinary heparin has a large molecule and this would tend to make its entrance into cells difficult. However, the fact that naturally-occurring heparins are frequently combined with lipids may be a factor of importance. Thus it is possible that the great effectiveness of the substance we obtain from the starfish ovary is due to the fact that it is a polysaccharide combined with a lipid. For it is well known that as a general rule substances soluble in fat solvents penetrate cells much more rapidly than those that are not soluble in fat solvents. A heparin-lipid compound injected into the blood of an animal would tend to enter the cells of the animal, and it might have less effect on the blood than it has on the cells it enters. Perhaps for practical purposes it is this type of compound we should seek to find. Certainly in various kinds of living tissues there must be many potent antimitotic substances of the sort we have postulated. We hope to continue our search for such substances.

SUMMARY

From several types of marine material, but especially from starfish ovaries, we have been able to extract with acidified sea water a substance that prevents the division of Chaetopterus eggs and sea-urchin eggs.

The active substance acts like heparin in keeping the protoplasm in the interior of the cells fluid; if present in sufficient concentration, it prevents the mitotic gelation.

The substance also causes a liquefaction of the cortex of the cell.

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A FIFTH ALLELE AT THE HISTOCOMPATIBILITY-2 LOCUS OF THE MOUSE AS DETERMINED BY TUMOR TRANSPLANTATION [1] [2] [3]

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According to the genetic theory of tumor transplantation, susceptibility to transplants is determined by multiple dominant genes (*1*). These genes have been called histocompatibility genes (*2*). Early studies were primarily concerned with proving the existence of such genes, and with providing an estimate of their number. The development of new inbred mouse stocks and the progress that has been made in recent years in locating mutant genes of the mouse in specific linkage groups have made it possible to design new methods adapted to the detection and analysis of specific histocompatibility loci (*2*). One such locus, designated as histocompatibility-2, has been found to belong to the ninth linkage group, in close association with the genes for fused tail (*Fu*), kinky tail (*Ki*) and brachyury or short tail (*T*), themselves all closely linked (*3*, *4*). Four alleles at this locus, *H–2*, *H–2*^{d}, *H–2*^{p}, and *H–2*^{b}, have already been described (*5*). These, together with the strains in which they are found, are listed in table 3. A preliminary note describing the fifth allele reported herewith has also been published (*6*).

MATERIALS AND METHODS

Eight inbred strains of mice have been used: A/Lilly, Ak, B alb C, CBA, C57BL/6, C57BL/10, dba/2, and P. The two sublines, 6 and 10, of the C57BL strain are closely related and have given identical results. Hence no distinction between them is made in the text or tables. Five noninbred stocks carrying the genes *Fu* or *Ki* have been employed. F^{h} is a fused stock derived from the CA stock which combined the genes *Fu*, *Ca*, and *W*. F^{H} is a stock derived from F^{h} by crossing over between the *Fu* and *H–2* loci following successive matings to the A strain. The *H–2* allele present is the same as that in the A strain. F^{c}, K8 and K9 are fused-tail and kinky-tail stocks, respectively (kindly provided by Dr. L. C. Dunn). Lines K8 and K9 are descended from noninbred sibs.

Two transplantable tumors were used, 15091a, an A strain tumor of

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mammary-gland origin, and S621, an induced fibrosarcoma of B alb C strain. All inoculations were subcutaneous, and made by the trocar method.

Most of the evidence in regard to alleles at the histocompatibility-2 locus was derived from crosses of the type

$$(M \times F) \times N$$

where M and N are any two inbred strains and F is a strain carrying *Fu* or *Ki*. The cross M × F was made first, and a fused tail (or kinky tail) F_1 was mated to strain N. The young from this second mating were classified as normal tail (+) or fused tail (*Fu*), and inoculated with tumor 15091a. Except for cases 1, 11, and 12 of table 1, this means that the tumor used in every case was native to the strain in the M position. Following inoculation, the mice were checked at approximately weekly intervals. They were classified as positive (+) if they succumbed to the tumor, as negative (−) if they survived.

In a second series of experiments, several groups of F_1 hybrids produced by crossing inbred strains were tested for susceptibility to tumors foreign to both parents.

RESULTS

The results are summarized in tables 1 and 2. Cases 5, 6, 7, 8, 9, 11, and 12 of table 1 are the critical cases for demonstrating the fifth allele, *H-2*^{k}. The other cases are included because they help to establish the validity of the method or because of their bearing on the occurrence of crossing over between *H-2* and *Ki*. The data from case 1 have been published elsewhere (*5*). The conclusions to be drawn from the crosses in table 1 can best be made clear by discussing each case individually.

Case 1.—The cross was (C57BL × F^c) × C57BL, inoculated with A strain tumor 15091a. Thirteen fused mice succumbed to the tumor, and 19 of 23 normal-tailed mice were negative. There is clear linkage between the fused gene and the gene determining susceptibility. The 4 normal-tailed susceptible (++) mice may have been crossovers, but in view of the known tendency of *Fu* to fail sometimes to express itself, it is more likely that most or all of them were normal overlaps. Another possibility is that they were genetically resistant mice which, for unknown reasons, succumbed.

The susceptibility of all *Fu* mice proves that either the *Fu* gene from the F^c stock, or a gene closely linked with it, produces susceptibility to A strain tumor 15091a. Data will be published elsewhere showing that crossing-over occurs between *Fu* and the factor for susceptibility. Hence, we may postulate a gene linked with *Fu* that causes susceptibility to 15091a. This gene we shall call *H-2*.

It is of interest to note that the inoculated mice from this cross had an A strain great-grandparent, but no other A ancestry. The genetic background was ⅞ C57BL. In this background at least, the gene *H-2* is sufficient as well as necessary for the growth of A strain tumor 15091a.

TABLE 1.—*Outcome of tumor inoculations in mice from crosses involving the genes fused* (Fu) *and kinky* (Ki). *All mice were inoculated with A strain tumor 15091a. Mice were classified as + if they succumbed to the tumor, as − if they survived*

Case No.	Cross	Normal tail		Fused tail (or kinky tail)		Conclusion
		+	−	+	−	
1	(C57BL × F^e) × C57BL	[1] 4	19	13	0	F^e is *H–2*. C57BL is not *H–2*; is $H\text{–}2^b$.
2	(A × F^H) × C57BL	18	0	6	0	F^H is *H–2*.
3	(A × K9) × C57BL	9	0	1	12	K9 is not *H–2*.
4	(A × K9) × CBA	5	0	0	8	K9 is not *H–2*. CBA is not *H–2*.
5	(A × K8) × P	14	[2] 2	0	6	K8 is not *H–2*.
6	(A × K8) × C57BL	5	[2] 1	0	3	K8 is not *H–2*.
7	(A × K8) × Ak	14	0	0	9	K8 is not *H–2*.
8	(A × K8) × dba/2	17	0	18	1	K8 is $H\text{–}2^k$.
9	(A × K8) × B alb C	14	0	11	0	K8 is $H\text{–}2^k$.
10	(A × K9) × dba/2	2	[2] 1	0	3	K9 is not $H\text{–}2^k$?.
11	(B alb C × F^e) × C57BL	[1] 6	21	10	0	$H\text{–}2^k \neq H\text{–}2^b$.
12	(B alb C × F^b) × P	[3] 6	13	0	13	$H\text{–}2^k \neq H\text{–}2^p$.

[1] Probably genetically *Fu* (normal overlaps), though occasional mice in this group may be crossovers, or genetically resistant mice that succumbed to the tumor.
[2] Tested genetically, not normal overlaps. May be crossovers.
[3] These mice succumbed to the tumor but survived longer than is the rule with mice susceptible to 15091a. They were probably genetically resistant. For an analysis of similar cases see (4).

TABLE 2.—*Growth of tumors 15091a and S621 in F_1 hybrid mice. Mice are classed as + only if they succumbed to the tumor*

Case No.	Cross	Tumor	Origin	Results	
				+	−
1	B alb C × C57BL	15091a	A	1	22
2	A alb C × P	15091a	A	1	12
3	B alb C × CBA	15091a	A	28	0
4	A/Lilly × P	S621	B alb C	0	6
5	A/Lilly × C57BL	S621	B alb C	0	6

This cross also proves that strain C57BL carries some allele other than *H-2*, for if C57BL's were *H-2/H-2 all* offspring from the cross would be susceptible. This fact has been established by data already published, and the allele in strain C57BL has been assigned the symbol *H-2*^{b}.[4]

The genotypes of the mice in the final cross in case 1 were thus: *H-2*^{b}+/*H-2Fu* × *H-2*^{b}+/*H-2*^{b}+.

Case 2.—The cross was (A × F^H) × C57BL. All mice from this cross were susceptible to A strain tumor 15091a. The indicated genotype of the animals in the final cross is *H-2*+/*H-2Fu* × *H-2*^{b}+/*H-2*^{b}+. Other data not given here strengthen the conclusion that the F^H stock is *H-2*. This cross is interesting by way of contrast with the following 5 crosses, which were rather similar except that the marker gene, instead of carrying in a gene for susceptibility to 15091a, carried in a gene for resistance to this tumor.

Case 3.—The cross was (A × K9) × C57BL. Since 12 of 13 *Ki* mice survived the tumor, the *Ki*-tagged chromosome must carry some allele other than *H-2*.

Case 4.—(A × K9) × CBA. All *Ki* mice survived. The conclusion is the same as in case 3. One new fact is demonstrated: That strain CBA is not *H-2*. If it were *H-2*, all mice would succumb.

Case 5.—(A × K8) × P. The survival of *Ki* mice demonstrates that K8, like K9, is not *H-2*.

Of the 16 normal-tailed mice, 14 succumbed and 2 survived. The survivors were tested genetically and demonstrated not to be normal overlaps (genetically *Ki* but phenotypically normal). They were not reinoculated, and there is a remote possibility that their survival was the result of faulty inoculation, but we have found this to be a rare occurrence. It seems more probable that they were genetically resistant. Such resistance might be due to modifying genes or to crossing over between *Ki* and *H-2*. Cases 1 and 2, where all of 37 genetically susceptible mice succumbed, do not point to the existence of modifying factors capable of causing survival. We have other data of the same sort, though case 8 produced one apparent exception. The 2 animals in question can prob-

[4] In previous publications, the symbol for the allele in the C57BL strain was written *h-2*^{b}. The small letter was selected because antibodies were not detected when C57BL tumors were grown in A mice, suggesting a recessive condition for this allele. They were detected in the reciprocal, combination (*A*). Transplant studies, however, indicate that all alleles express themselves in the heterozygote. They should therefore be regarded as dominant and symbolized by a capital letter.

ably be interpreted as crossovers, though conclusive evidence on this point can only be provided by more extensive tests.

Case 6.—(A × K8) × C57BL. Same results and same conclusion as case 5. There is one more probable crossover.

Case 7.—(A × K8) × Ak. Same results and same conclusions as cases 5 and 6.

Case 8.—(A × K8) × dba/2. All of 17 normal-tailed and 18 of 19 kinky-tailed mice succumbed. If this cross were taken by itself, the obvious interpretation would be that K8 carries the allele *H-2*. However, we have just proved by cases 5, 6, and 7 that it does not carry the allele *H-2*. Strain dba/2, together with strain B alb C, is known from previous data to carry an allele $H\text{-}2^d$, which by itself does not produce susceptibility to strain A tumor 15091a. The only possible conclusion would appear to be that the K8 line carries an allele, $H\text{-}2^k$, such that heterozygous mice of the genotype $H\text{-}2^k/H\text{-}2^d$ are susceptible to 15091a.

A parallel case occurs in the Rh series of alleles (or linked loci) in man (*7*). Here, too, certain heterozygous combinations of alleles are the equivalent of a third allele. In the mouse the two genotypes A^w/a and A/a^t are indistinguishable, both being light-bellied agouti. There is thus good precedent for the proposed interpretation of this case.

The genotype of the animals used in the final cross is therefore $H\text{-}2+/H\text{-}2^k Ki$ $H\text{-}2^d+/H\text{-}2^d+$.

Case 9.—(A × K8) × B alb C. As mentioned above, B alb C resembles dba/2 in that it carries the allele $H\text{-}2^d$, though the strains are otherwise unrelated. The cross should give the same results as case 8, and does in fact do so. This confirms the conclusion that K8 carries a distinct allele, $H\text{-}2^k$.

Case 10.—(A × K9) × dba/2. Here all *Ki* mice survived, but the numbers are small, and the K9 line has subsequently been lost. Presumably K9 carried an allele that was neither *H-2* nor $H\text{-}2^k$. The significance of this fact is that *Ki* can be associated with more than one type of factor for susceptibility. This does not rule out the possibility that the *H-2* locus and the *Ki* locus are one and the same, with a number of alleles all producing the same tail manifestation but different susceptibility manifestations, but the occurrence of distinct loci separable by crossing over would appear the most probable interpretation.

Case 11.—(B alb C × F^c) × C57BL. Mice from this cross were inoculated with A strain tumor 15091a. The purpose of this cross was to determine whether allele $H\text{-}2^k$ found in line K8 and allele $H\text{-}2^b$ characteristic of the C57BL's (*5*) are nonidentical. If the C57BL allele were $H\text{-}2^k$, the normal-tailed mice from this cross would be $H\text{-}2^d/H\text{-}2^k$ and should succumb. Actually 21 of 27 normal-tailed mice survived. The 6 that succumbed were probably normal overlaps, as these occur rather frequently in this type of cross. Hence we may conclude that $H\text{-}2^k \neq H\text{-}2^b$.

Case 12.—(B alb C × F^{h}) × P. Strain P has previously been reported (*5*) as carrying a distinct allele, *H-2^{p}*. This cross, in the same manner as cross 11, proves that *H-2^{k}*≠*H-2^{p}*.

H-2^{k} is therefore distinct from all previously described alleles and may be regarded as allele number 5.

Table 2 contains additional data pertinent to allele *H-2^{k}*.

The first two crosses, B alb C × C57BL and B alb C × P, inoculated with A strain tumor 15091a, confirm the conclusion already reached that *H-2^{k}*≠*H-2^{b}* or *H-2^{p}*. If either of these alleles were identical with *H-2^{k}*, we should expect the offspring of the respective cr ·s to be susceptible, whereas actually the offspring in both cases were resistant.

Several stocks with unidentified *H-2* alleles were tested by crossing to B alb C or dba/2 to see if they gave F_1's susceptible to 15091a. Case number 3 of table 2 shows that CBA × B alb C does give such young. Though both parents are resistant to 15091a, the hybrids are susceptible, with growth being essentially as rapid as in pure A mice. The allele in strain CBA may be tentatively identified as *H-2^{k}*. Further tests are in progress.

Cases 5 and 6, table 2, are two crosses, with strain A as one parent, in which hybrids were inoculated with B alb C tumor S621. These tests were suggested by the antigenic relationship between strains A and B alb C indicated by the growth of 15091a (A strain) in *H-2^{k}*/*H-2^{d}* hybrids. Results were negative in each case.

Table 3 summarizes the present state of information in regard to *H-2* alleles.

DISCUSSION

We have shown that mice of the genotypes *H-2*/*H-2*, *H 2*/— and *H-2^{d}*/*H-2^{k}* react identically to A strain tumor 15091a. This is ground for supposing that allele *H-2*, characteristic of the A strain, is composed of (or produces) two components (antigens?), d and k. Actually the symbol for this allele might well be written *H-2^{dk}* (or *H-2^{DK}*), but it is probably wisest to let any revision of the nomenclature wait until tests for other possible alleles at this locus are completed. There is of course the possibility that we may be dealing with two or more closely linked loci rather than with a single locus.

SUMMARY

Previous work has demonstrated the existence of four alleles, *H-2*, *H-2^{d}*, *H-2^{b}*, and *H-2^{p}*, at the histocompatibility-2 locus in the mouse. This locus is on the 9th chromosome, closely linked with the genes *Fu*, *Ki*, and *T*. This paper reports a fifth allele at this locus, *H-2^{k}*.

Most of the evidence was obtained from crosses of the type (M × F) × N, where M and N are any two inbred strains, and F is a strain carrying *Fu* or *Ki*. Mice from the double cross were, in most cases, inoculated with a tumor from the strain in the M position. It can be shown by simple genetic reasoning that where, in such a cross, susceptibility is linked with normal tail and resistance with fused or kinky tail (*Fu* or *Ki*),

strains M and N must carry *different H-2* alleles. By repeating the test with different inbred strains in different combinations, new alleles, where they exist, can be identified.

Allele *H-2*^{k} is unusual in that mice of the heterozygous genotype *H-2*^{k}/*H-2*^{d} will grow A strain (*H-2*/*H-2*) tumor 15091a. It is concluded that the allele *H-2* may possess a d and a k component.

Table 3 summarizes the known alleles and the strains in which they are found.

TABLE 3.—*Distribution in standard inbred strains and in stocks with ninth chromosome marker genes of alleles at the* H-2 *locus*

Allele	*H-2*	*H-2*^{d}	*H-2*^{b}	*H-2*^{p}	*H-2*^{k}
Stock	A F^c F^H	dba/2 B alb C	C57BL/6 C57BL/10	P	K8 CBA?

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